Atty. Dkt. No. 016777/0309

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: Kaare M. GAUTVIK et al.

Title: *Production of Human Parathyroid Hormone From Microorganisms*

Appl. No.: 09/287,332

Filing Date: April 7, 1999

Examiner: R. Landsman

Art Unit: 1647

**DECLARATION OF KAARE M. GAUTVIK, M.D.**

**PURSUANT TO 37 C.F.R. § 1.132**

Mail Stop NON-FEE AMENDMENT
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, KAARE M. GAUTVIK, declare as follows;

1. I am a co-inventor of the above-captioned application.

2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as exhibit A.

3. The standard hPTH(1-84) referred to on page 7, line 19, of the specification is synthetic hPTH obtained from chemical supply companies, including Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem.

4. I participated in the experiment detailed below to study the purity of the synthetic hPTH obtained from Peptide Institute Protein Research Foundation, Penninsula

Laboratories, Sigma, and Bachem as compared to recombinant hPTH produced according to the claimed invention. The experiment was performed on November 30, 1989 and the commercial hPTH preparations were purchased from 1986-1989.

**Experimental Method**

5. 0.2 μg of hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem, and from hPTH(1-84) produced according to the claimed invention, was analyzed on an SDS-PAGE gel. The protein bands were stained by silver nitrate. Silver nitrate is the most sensitive stain to disclose impurities. The hPTH(1-84) was loaded onto the gel as follows. Where available, lot numbers, catalog numbers, and date of arrival to the laboratory are provided.

Lane 1: BRL, low molecular weight standard

Lane 2: Peptide Institute Protein Research Foundation, lot no 350306, arrived July 1986

Lane 3: Penninsula Laboratories, arrived July 27th 1986

Lane 4: Sigma P-7036, arrived before October 1st, 1987

Lane 5: Bachem, lot no ZF567, arrived July 1989

Lane 6: Bachem, lot no 734B, arrived February 1989

Lane 7: *E.coli* PTH (recombinant) (produced according to claimed invention)

Lane 8: Yeast PTH (recombinant) (produced according to claimed invention)

Lane 9: Yeast (Q26) PTH (recombinant) (produced according to claimed invention)

Lane 10: BRL, low molecular weight standard

**Results**

6. A photograph of the SDS-PAGE gel obtained from the above-described experiment is provided as Exhibit B. Since the lanes of the gel were loaded with the same amount of hPTH(1-84), 0.2 μg, according to the manufacturer's description, the intensity/size of the bands should be the same. However, as shown in the SDS-PAGE gel picture attached as Exhibit B, the intensity/size of the bands is not the same for the hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem (lanes 2-6), as compared to the recombinant hPTH produced according to the claimed invention (lanes 7-9). Further, impurities (shadows appearing above or below the main band) are apparent in the lanes containing hPTH(1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem, but are virtually absent in the lanes containing recombinant hPTH produced according to the claimed invention. A more detailed analysis of the results of the gel is provided below.

7. Lane 2: The molecular weight of the main band is higher than the expected molecular weight for hPTH(1-84). Impurities are seen on the photograph above and below the major band. The material in lane 2 contains less hPTH(1-84) than the manufacturer's stated quantity.

8. Lane 3: The molecular weight of the main band is higher than the expected molecular weight for hPTH(1-84). Impurities are seen on the photograph above and below the major band. The material in lane 3 contains less hPTH(1-84) than the manufacturer's stated quantity.

9. Lane 4: Low molecular weight impurities are present. The material in lane 4 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

10. Lane 5: Low molecular weight impurities are present. The material in lane 5 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

11. Lane 6: Low molecular weight impurities are present. The material in lane 6 contains less hPTH(1-84) than the manufacturer's stated quantity. The impurities lead to false weight estimations.

12. Lane 7: The hPTH(1-84) is more than 95% pure hPTH(1-84) of the correct molecular weight. There is virtually no material above or below the major protein band.

13. Lane 8: The hPTH(1-84) is more than 95% pure hPTH(1-84) of the correct molecular weight. There is virtually no material above or below the major protein band.

14. Lane 9: QPTH moves a little faster than hPTH(1-84) because of one amino acid residue substitution, and there is no sign of impurities

15. Although the synthetic hPTH (1-84) obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem was impure, this synthetic hPTH (1-84) was used as a hPTH (1-84) standard in my lab. The impure hPTH (1-84) standard was used to confirm in early experiments the identity and characteristics of hPTH (1-84) produced according to the claimed invention. The impurities present in the synthetic hPTH (1-84) standard did not as such, affect the usefulness of the synthetic hPTH (1-84) as a qualitative "standard."

16. As a consequence of impurities and incorrect amounts provided by the commercial producers, the specific biological activity of even their best preparation was found to be about 30% below that of the recombinant hormone. (*See,* e.g., exhibit C, Reppe, S., et al., 1991, JBC, 22:14198-14201, Figure 5).

17. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 12.07.04

KAARE M. GAUTVIK, M.D.

June 2003

# CURRICULUM VITAE
# RELEVANT PUBLICATIONS
## KAARE M. GAUTVIK, M.D., PHD, CHIEF CONSULTANT

**Personal and marital status:**


| | |
|---|---|
| Name: | **Kaare M. Gautvik** |
| Home address: | Bregnevn. 3, 0875 Oslo, Norway |
| Business address: | Institute of Medical Biochemistry, University of Oslo, P.O.Box 1112 Blindern, 0317 Oslo, Norway |
| Telephones: | 47-22851055 (work); 47-22235137 (home) |
| Date and place of birth: | 11th of December 1939 in Oslo. |
| Social Security: | No.: 111239.39311 |
| Married to: | Vigdis Teig Gautvik, date of birth: 24th of March 1947 |
| Children: | Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964 Silja Marie Sakrisvold Gautvik, date of birth: 31th of March 1973,Ole Martin Teig Gautvik, date of birth: 21th of January 1982 |


**Education and Clinical Specialities:**

1. August 1958-June 64, Medical School at the University of Oslo.
2. 1967-69, Courses in mathematics involving geometry, statistics and mathematical analysis.
3. May 1970, Disputation for the medical doctor degree at the University of Oslo.
4. 1985, Specialist in clinical chemistry, and physiology and nuclear medicine.
5. 1986, Specialist in occupational health medicine.

**Employment:**

1. June 1964-June 1965, working at Tromsø University Hospital at medical and surgical departments.
2. July 1965 until December 1965, working as a general practioner in Sjøvegan, Troms.
3. One year military service as a major in The Norwegian Air Force, working mainly at the Norwegian Institute for Aviation and Space Medicine.
4. From 1967, position as post-doctoral researcher at The Institute of Physiology, University of Oslo.
5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973-89.
7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
   (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).
9. From Jnuary 2002 employment as senior consultant at Department of Clinical Chemistry, Labaratory Division, Ulleval University Hospital and professor II at the University of Oslo.

**Post-doctoral training abroad:**

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.
5. 1997, 3 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

**Teaching responsibility:**

1. One year teaching in aviation medicine for medical personnel and pilots.
2. I have given lectures and courses for medical students in following subjects:
   Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology.
   From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3. Organized interfaculty advanced courses within molecular endocrinology.
4. Lectures have been given in the following subjects at post-doctoral courses:
   Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5. Invited lectures: Several places in the U.S., In Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.

6. Chief organizer of post graduate scientific courses for the Medical Faculty at University of Oslo, 1986-1991.
7. Organizer of international scientific meetings within the frame of the following societies: Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below:

**Examples of specially invited symposium lectures:**

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.
2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.
3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.
4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).
5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.cerevisiae studied by in vitro mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.
6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).
7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Maccelesfield SK10 4TJ, UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal β-oxydation, enzyme activities and mRNA levels".
8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning and expression of human parathyroid hormone in microorganisms".
9. Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could not attend).
10. May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biology".
11. January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA. Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of G-coupled hormones".
12. August 1993: Invited by Norwegian Society of Charted Engineers, The Blindern Conference. Lecture entitled: "Experience from industrializing basal research".
13. November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at "Graduate course in molecular endocrinology - a problem oriented approach". The lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".
14. February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in microorganisms and animal cells with special reference to signal sequence efficacy and intracellular modifications".
15. September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology, Madurai Kamaraj University, India, to held a lecture in the symposium "Gene expression systems", XVIth IUBMB, New Delhi. Lecture entitled: "Expression of human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a milk protein in transgenic mice".
16. November 1994: Invited by Professor A. Taschjian Jr., Harvard School and Public Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and functional characteristics of the human TRH receptor cDNA and mapping of the gene".
17. February 11-13, 1995: Cairns, Australia, Workshop on "Animal models in the prevention and treatment of osteopenia".
18. February 1995: Int. Meeting of Calcified tissue research, Melbourne, Australia.
19. May 1996: Dublin University Program. "How to identify patients at risk for development of ostoporosis".
20. September 1996: Lecture at Scripps Research Institute, San Diego. "Unique hypothalamic specific mRNAs expressed by molecular subtraction hybridization".
21. September 1996: Invited seminar at the Astra Research Center, Montreal. "Cloning and expression of human polypeptide hormones with biomedical potential".
22. November 1996: Invited lecturer, The Norwegian Rheumatological Society, Oslo, "PTH (parathyroidea hormone) - The biochemical foundation for treatment of osteoporosis".
23. December 1996: Invited at Nordic Conference for Medical Treatment of Osteogenesis Imperfecta, Holmen Fjordhotel, Asker, Norway. "Characteristics of bone remodelling in patients with osteogenesis imperfecta".

24. January 1997: Invited lecturer at The Salgrenska Hospital in Sweden. "Characterization and functional analysis of novel hypothalamus genes as identified by directional tag substraction".
25. 1998: Guest lecturer at Scripps Research Institute: "Hypothalamic calcium-calmodulin kinase-cloning and functional aspects".
26. February 1999: Only invited speaker from abroad at National Osteoporosis Congress in Rio de Janeiro, Brazil.
27. 2000: Lecture at NPS-Allelix company and Toronto University: "Parathyroid hormone regulated bone remodelling".
28. May 2000, Rio de Janeiro, Brazil. Member of the International Scientific Panel at the International Congress in Osteoporosis.

**Honorary lectures and prizes:**

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".
2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.
3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.
4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.
5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.
6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.
7. Given a 3 years economical "Group Research Support of 0.8 M NOK per year from 1997" after national and international project evaluations.
8. Scientific prize (Abstract award) 1997, at the Endocrine Society "Cloning and Organization of the human TRH-receptor Gene".
9. Norwegian Endocrine Society Prize (S.Reppe) for "Sox4 – a PTH regulated transcription factor in bone".
10. Endocrine Society 1998: Poster Award: Cloning and regulation of the thyroliberin receptor gene.
11. Norwegian Endocrine Society prize 1998: Hormone regulated bone remodelling.
12. American Society for Bone and Mineral Research (ASBMR); 1999 Best Poster Award: The Transcription factor Sox-4 is expressed in developing cartilage and bone cells.

**Consulting appointments:**

1. Senior honorary consultant for NPS Biotechnology, Salt Lake City, Colorado, USA.
2. Consultant for Karolinska Institute, Stockholm, Sweden.

**Referee activity:**

I am or have been working as referee for the following international journals:
Endocrinology,J. Expl. Cell Res.,Acta Physiol. Scand. (Kbh.),Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.),Eur.J.Clin. Invest.,Hormone Research,Acta Obstet. Gynecol. Scand.,Journal of Endocrinological Investigation,Eur. J. Biochem.,Experimental Cell Research,J. Biol. Chem.

**Guidance for the academic doctor degree:Twenty three and 5 ongoing.**
**Supervision of postgraduate candidates: Presently** three.
**Supervision of students' main degrees:** Nineteen.
**Guest research workers from abroad:**In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.
**Member of committees for the academic doctor degree in Norway and abroad:** 15.
**Member of advisory international/national committees for evaluation of professor positions:**14.
**Honorary Societies:**Member of the Norwegian National Academy of Science and Letters
**Professional memberships:**Norwegian Society of Biochemistry,Norwegian Society of Physiology,Norwegian Society of Endocrinology,Endocrine Society (USA),American Society for Bone and Mineral Research (USA)
**Medical Faculty Responsibilities:**

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Research Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's councel for evaluation of postgraduate applications from 1989-1993.
6. Committee member of the Medical Faculty's Scientific Instrument Board, 1996-.
7. Committee for Medical Research collaboration and interaction between University of Oslo and the National Hospital, 2000-.

**National- and International Research Council Responsibilities:**

1. Leader of Chemical Peptide Synthesis Core facility 1984-1989.

2. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
3. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
4. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
5. Member of the Premedical Institute Group Committee for Preclinical Sciences from 1989-2003.
6. Member of the International Scientific Board of Novo-Nordisk Research Committee, 1989-2001.
7. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway, 1989-present.
8. Chairman of the Research Council in the Norwegian Association for Osteoporosis, 1993-2003.
9. Leader of DNA Sequencing Core facility of the Institute of Basic Science, 1999-present.

*10. Consultant and peer reviewer within Wallenberg Consortium North Technology Platforms DNA; SNP (single nucleotride polymorphism) Technologies and the Platform for Proteomics on behalf of the Board of the Wallenberg Consortium North, Stockholm, Sweden, 2001-2004.*

*11. Coordinator for Marie Curie Training Sites Fellowship No MCFH-200-00040 :"Oslo Doctoral Training Site for Diagnosis and Therapy of Osteoporosis"—2001-2005.*

*12. Coordinator of EU 6.Program STREP contract no 502941, "Molecular mechanisms of bone homeostasis" (OSTEOGENE). Eight partners in 5 countries- 2003-2006*

**Awards and fellowships:**

1967, 3 months, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.

1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.

1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.

1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

1997 Awarded for 3 years the Norwegian Research Council's Science Prize for outstanding research.

*2001 Promoted by EU to become Oslo Doctoral Training Site for Diagnosis & Therapy Of Osteoporosis, received by a group consisting of scientists from University of Oslo, IMBA and the National Hospital.*

*2002 Member of CNS Molecular Biology group (leader Ivar Walaas) appointed as a "Research Theme Priority" at the Medical Faculty,2002- 2007*

*2004 OSTEOGENE (Molecular mechanisms of bone homeostasis ) project given the highest priority and the only selected for presentation within Health Region East 2004.*

**Other professional activities:**

1. Founder of the Norwegian Association for Osteogenesis Imperfecta 1978 (Norsk Forening for Osteoporosis Imperfecta) together with Mrs. L. Myhre.
2. Founder of the Norwegian Association for Osteoporosis (Norsk Osteoporoseforening) 1993, together with Norwegian Women Public Health Association (NKS).

**Patents:**

I.Two U.S. patents, U.S. Patent No. 5.010 010 and No. 5.420.242 are held with international extensions in Europe, Japan, Canada, and Australia. In addition, three Divisional Applications are submitted to the U.S. Patent Office and elsewhere.These patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms for the use in treatment of osteoporosis.

II.Inventor in patent application from Scripps Research Institute on: Novel hypothalamic mRNAs, the corresponding peptides and their functions.

**Publications: *More than 200 original articles published in internationally well reputated and refereed journals. Relevant articles are cited in relation to description of the research activities :***

## A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS AND RELEVANT REFERENCES

### A.STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The results so far from our refinement and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method have been successful. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. We have so far described several novel peptides: hypocretin (the cause of Narcolepsy) and several very interesting peptides, e.g. novel CaM kinase (see list of references). Another CNS peptide is somatostatin-like, called cortistatin, structure similarity with somatostatin; P25 and Vat 1 , two uniquely expressed peptides in distinct regions of the brain.

1.Gautvik, K.M., de Lecea, Luis, Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. Overview of the most prevalent hypothalamus-specific mRNAs identified by directional tag PCR subtraction.
Proc. Natl. Acad. Sci. USA (PNAS) 93: 8733-8738, 1996.

2.de Lecea, L., Criado, J.R., Prospero-Carcia, O., Gautvik, K.M., Schweitzer, P., Danielson, P.E., Dunlop, C.L.M.Siggin G.R., Henriksen, S.J. and Sutcliffe, J.G. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381: 242-245, 1996.

3.de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F.,Gautvik, V.T., Bartlett II, F.S., Frankel, W.N., Van den Pol, A.N., Bloom, F.E., Gautvik, K.M. and Sutcliffe, J.G. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA (PNAS) 95: 322-327, 1998.

B. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE BONE CELL PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described above, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual cones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumor type ( Olstad et al. ,2003)

C.ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS ("Bone anabolic genes")

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of sentral importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular substraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is almost completed in a highly successful manner. We have isolated more than 40 genes which are involved in PTH anabolic action in bone, and among those we are searching for the gene(s) causing postmenopausal osteoporosis.

D. As a complementation an to the activities described above, we have embarked on defining the bone phenotype in female and male osteoporosis within the context of the EU project OSTEOGENE (see above). About 100 patients and controls will have bone biopsies which will be prepared and analysed for their global gene expression and differences at the micro- and ultrastructural level. I am the coordinator of this activity including 5 countries and where Oslo university and three hospitals( Ullevål university hospital,the National Hospital and Lovisenberg hospital are working closely together. This is a direct consequence and follow up of previous research representing patient related basic and translational science aiming to solve the mechanisms of osteoporosis, the most common disease in women of 50 yrs of age.

**THE MAIN RESEARCH ACTIVITIES DURING THE LAST 8 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:**

I. PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

i) Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.
ii) Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

1.Høgset, A., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Jacobsen, P.B., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression of human parathyroid hormone in Escherichia coli. BBRC 166: 50-60, 1990.

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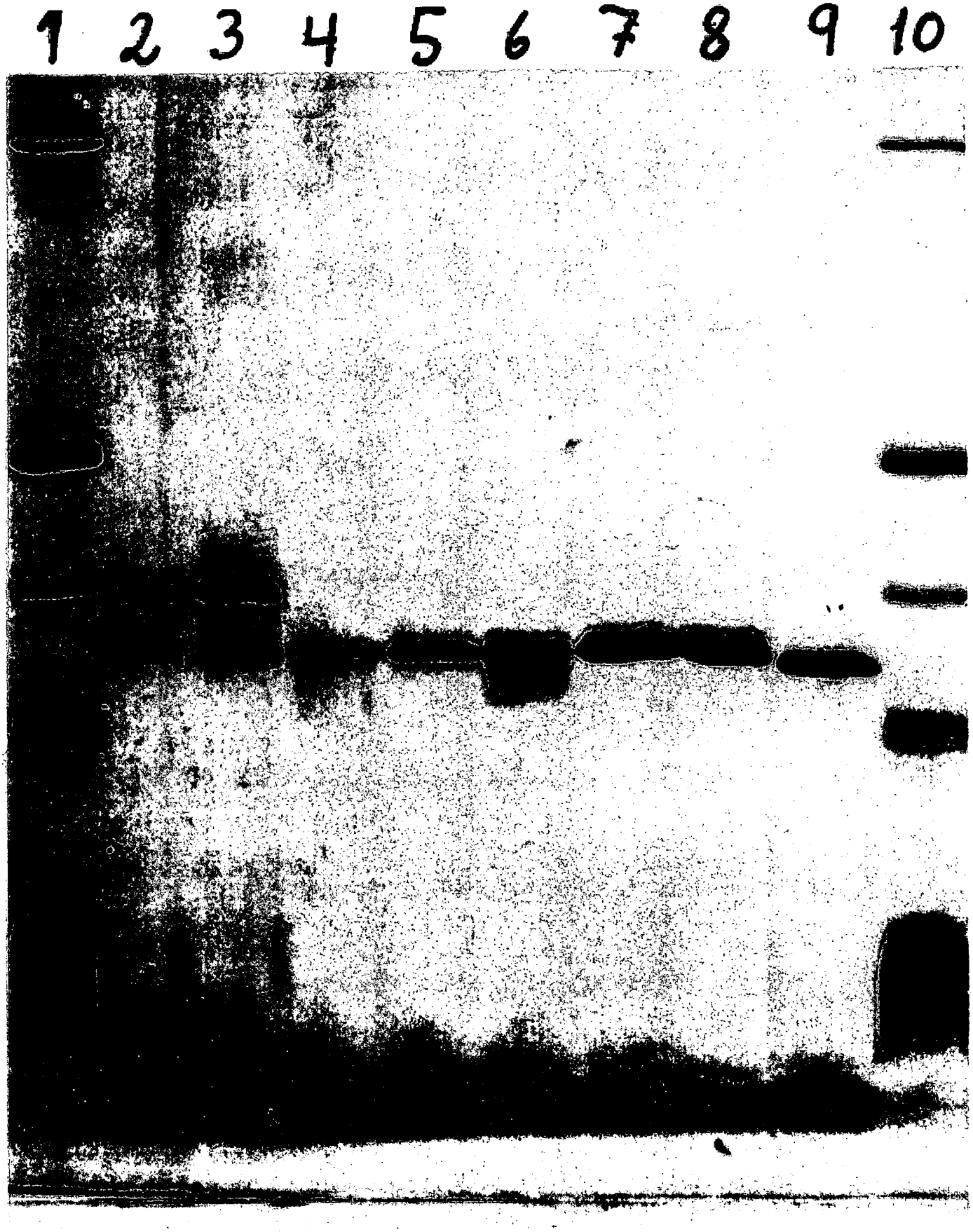
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# Characterization of a K26Q Site-directed Mutant of Human Parathyroid Hormone Expressed in Yeast*

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**Human parathyroid hormone (hPTH) is susceptible to proteolytical cleavage both in humans and when expressed as a secretory product in *Escherichia coli* (Høgseth, A., Blingsmo, O. R., Sæther, O., Gautvik, V. T., Holmgren, E., Hartmanis, M., Josephson, S., Gabrielsen, O. S., Gordeladze, J. O., Alestrøm, P., and Gautvik, K. M. (1990) *J. Biol. Chem.* 265, 7338–7344) and *Saccharomyces cerevisiae* (Gabrielsen, O. S., Reppe, S., Sæther, O., Blingsmo, O. R., Sletten, K., Gordeladze, J. O., Høgset, A., Gautvik, V. T., Alestrøm, P., Øyen, T. B., and Gautvik, K. M. (1990) *Gene (Amst.)* 90, 255–262). In the latter system, one major site of cleavage was identified ($Arg^{25}$-$Lys^{26}$↓$Lys^{27}$). To produce hPTH resistant to this proteolytic processing, a point mutation changing $Lys^{26}$ to Gln was introduced, and the modified gene expressed in *S. cerevisiae* as a fusion protein with the α-factor leader sequence. The resulting major form of hPTH secreted to the growth medium was of full length showing that the mutation had eliminated internal processing. Consequently, the yield of the mutant hormone was significantly higher than obtained with the natural peptide. Using improved purification procedures, a significantly higher purity was also obtained. The secreted mutant hPTH-(1–84,Q26) had the correct size, full immunological reactivity with two different hPTH antisera, correct amino acid composition and N-terminal sequence, and correct mass as determined by mass spectrometry. Furthermore, the introduced mutation did not reduce the biological activity of the hormone as judged from its action in three biological assay systems: 1) a hormone-sensitive osteoblast adenylate cyclase assay; 2) an *in vivo* calcium mobilizing assay in rats; and 3) an *in vitro* bone resorption assay.**

Gene fusions with the mating pheromone α-factor leader sequence have been widely used to express, correctly process, and efficiently secrete human proteins of medical interest in *Saccharomyces cerevisiae* (1–4). Contrary to the situation in higher eukaryotes where the propeptide processing signal seems to involve proximal residues in addition to the doublet of basic amino acids (5, 6), there is no evidence that yeast yscF endopeptidase (*KEX2* gene product) requires more than a pair of basic amino acids to process a propeptide, although a Lys-Lys pair is a poor substrate for the enzyme (6, 7). Since the probability of finding two consecutive basic amino acids (Lys-Arg, Arg-Lys, or Arg-Arg) in a random protein sequence is rather high, many heterologous proteins risk to be aberrantly processed during secretion in yeast. Thus, in several cases where such sites have been present in the mature peptides, the secretion products were reported to be either partially (8) or completely (9) cleaved, heterogeneous (10), or to contain extra unidentified peptides (11), suggesting aberrant processing.

The mature human parathyroid hormone (hPTH)[1] illustrates this problem because it contains two potential processing sites (Arg-Lys) in the 84-amino acid-long molecule. Hence, when the hPTH gene, fused to the α-factor leader, was expressed in *S. cerevisiae*, a significant fraction of the synthesized protein was cleaved at the first of these sites. The cleavage site was localized after the two basic amino acids ($Arg^{25}$-$Lys^{26}$↓$Lys^{27}$), suggesting that the yscF protease is involved (12).

In the present report we describe the production in yeast of a mutant hPTH where the internal putative yscF cleavage site has been removed by *in vitro* mutagenesis, substituting lysine at position 26 ($Lys^{26}$) by glutamine ($Gln^{26}$). As a consequence, the major degradation product hPTH-(27–84) (12) was no longer found in the growth medium while the yield of full-length hormone increased. The secreted hPTH-(1–84,Q26) had correct size, mass, sequence, and immunoreactivity. Furthermore, the introduced mutation did not reduce the biological activity of the hormone as judged from its action in several biological test systems.

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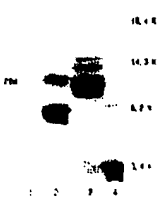
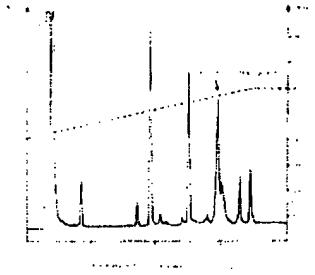
[1] The abbreviations used are: hPTH, Human parathyroid hormone; hPTH-(1–84), full-length (84 amino acids long) human parathyroid hormone with no mutations; hPTH-(1–84,Q26), full-length human parathyroid hormone where amino acid $Lys^{26}$ is mutated to $Gln^{26}$; prefix y, produced in yeast (*S. cerevisiae*); prefix s, synthetic (commercial preparation).

**FIGURE 1.**



SDS PAGE of concentrated yeast growth medium containing mature and non-mature hPTH. Aliquots of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids pαIXPTH-Q36 (lane 3) and pαIXPTH-2 (12) (lane 2) were analysed by 15% PAGE in the presence of 0.1% SDS, and visualised by silver staining as described in Materials and Methods.
Lane 4: molecular size markers.
Lane 1: a commercial hPTH standard (Bachem).

**FIGURE 2A.**



HPLC purification of yeast derived hPTH(1-84,Q26). Yeast medium concentrated on S Sepharose FF was further purified as described in Materials and Methods. The elution profile from the first HPLC step, a Vydac C18 column, is shown. The peak containing shPTH(1-84,Q26) is marked. Several peaks probably represent non-peptide low molecular weight substances from the fermentation broth since they do not give rise to bands when analysed by SDS-PAGE.

**FIGURE 2B.**

The elution profile from the second HPLC step, a Waters Nova-Pak C18 column, isocratic elution, with an analysis of the final purity of shPTH(1-84,Q26) (0.6 mg) as revealed by SDS PAGE and silver staining. Lane 2 shows shPTH(1-84,Q26) from the main HPLC peak. The molecular weight marker in lane 3 is the same as in Figure 1, but includes a reference hPTH produced in *E.coli*. Lane 1 shows a slower migrating hormone (0.6 mg) which is O-glycosylated (unpublished results, and that elutes in the minor front peak from the second HPLC.

**Table 1**

N-terminal amino acid sequence of shPTH(1-84,Q26).

```
                1                   5                  10                  15
Ser-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-Leu-
           20                  25                  30
Asn-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Gln-Lys-Leu-Gln-Asp
```

See Materials and Methods for experimental details.

**Table 2**

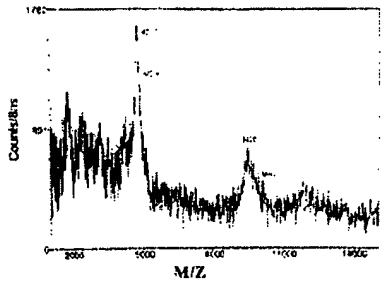
Total amino acid composition of shPTH(1-84,Q26).

| AA | shPTH(1-84,Q26): Found | Theory | shPTH(1-84): Found | Theory | shPTH(1-84)[1] Found | Theory |
|---|---|---|---|---|---|---|
| Asx | 10.2 | 10 | 10.1 | 10 | 10.4 | 10 |
| Glx | 11.8 | 12 | 10.7 | 11 | 10.8 | 11 |
| Ser | 7 | 7 | 7.3 | 7 | 7.4 | 7 |
| Gly | 4 | 4 | 4.4 | 4 | 4.3 | 4 |
| His | 3.7 | 4 | 3.8 | 4 | 3.7 | 4 |
| Thr | 1.2 | 1 | 1.2 | 1 | 1.1 | 1 |
| Ala | 6.9 | 7 | 6.7 | 7 | 6.8 | 7 |
| Arg | 5 | 5 | 5.4 | 5 | 5.4 | 5 |
| Pro | 3 | 3 | 3.2 | 3 | 3.0 | 3 |
| Val | 7.5 | 8 | 7.8 | 8 | 7.7 | 8 |
| Met | 1.9 | 2 | 1.8 | 2 | 1.7 | 2 |
| Ile | 0.9 | 1 | 0.9 | 1 | 0.8 | 1 |
| Leu | 9.5 | 10 | 9.6 | 10 | 9.7 | 10 |
| Phe | 1.2 | 1 | 1.1 | 1 | 1.1 | 1 |
| Lys | 7.6 | 8 | 8.7 | 9 | 8.8 | 9 |
| Trp | n.d. | 1 | n.d. | 1 | n.d. | 1 |
| Sum | 81.1 | 84 | 82.9 | 84 | 82.9 | 84 |

1) Bachem standard hPTH

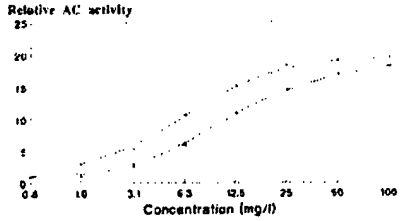
See Materials and Methods for experimental details.

**FIGURE 3.**




Plasma desorption mass spectrometric analysis of purified recombinant shPTH(1-84,Q26). The spectrum shows the ion abundance as a function of mass over charge.

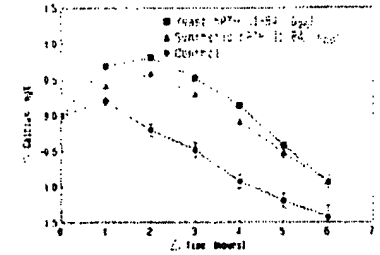
**FIGURE 4.**




Adenylate cyclase-stimulating activity of shPTH(1-84,Q26)

Purified recombinant hPTH(1-84,Q26) (—) was assayed for biological activity in a hormone-sensitive osteoblast adenylate cyclase (AC) assay as described in Materials and Methods. Relative AC activity means relative to control-activity set equal to 1. Each point gives the mean value of triplicate samples. hPTH(1-84) from Bachem (---) was used as reference.

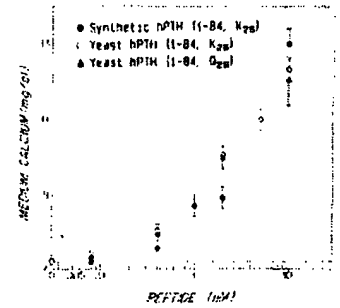
**FIGURE 5.**




*In vivo* induction of hypercalcemia by shPTH(1-84,Q26)

Parathyroidectomized male Wistar rats were dosed for induction of hypercalcemia by shPTH(1-84,Q26) and Bachem shPTH(1-84) standard as described in Materials and Methods. Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 hours after injection of PTH (2 μg of shPTH(1-84,Q26) and 2.75 μg of shPTH(1-84)). The induced hypercalcemia is given as change (Δ) in serum calcium concentration above the zero-time control value.

**FIGURE 6.**




*In vitro* bone resorption-stimulating activity of shPTH(1-84,Q26)

Dose response relationships for shPTH(1-84), yhPTH(1-84), and yhPTH(1-84,Q26) as bone resorption-stimulating agents. Each point gives the mean value and the brackets give the SE for groups of 6 to 28 bones. Results were obtained from up to 5 independent experiments and were pooled where statistically valid. The medium calcium concentration from control bones was 7.3 ± 0.08 mg/dl for 33 separate bones and is shown by the open square and dashed horizontal line. The treatment period was 72 hours.

MATERIALS AND METHODS AND RESULTS[2]

DISCUSSION

The hPTH appears to be especially sensitive to proteolytic degradation. Large amounts of hPTH fragments are found in the parathyroid gland (13), and only about 10% of circulating hPTH immunoreactive peptides are full-length hormone (14, 15). Similarly, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms (16, 17). Improved yields of intact hormone have been obtained by secretion of the hormone into the growth medium, either in *Escherichia coli* (18) or in *S. cerevisiae* (12) or by expressing hPTH as an intracellular fusion protein in *E. coli* (19, 20). Even in these systems, proteolytically cleaved forms may be produced reducing the yield of intact hormone.

The present results show that hPTH can be expressed in a correctly processed and secreted in a largely intact form in *S. cerevisiae* after the introduction of a single, structurally conservative mutation in the 26th amino acid ($Lys^{26} \rightarrow Gln$). The hormone produced resists the frequent cleavage found in the non-mutated hormone (12).

The increase in final yield from shake flask cultures of pure full-length hormone agonist is 5–10-fold compared with that obtained with the nonmutated hormone expressed in the same system (12) using identical culture conditions (results not shown). An improved purification procedure gave homogeneous hPTH-(1–84,Q26) after two high performance liquid chromatography steps.

The mutation was introduced in a region of hPTH previously found to be essential for biological activity (13) and within a triplet of basic amino acids conserved between the rat (21), porcine (22), bovine (23), and human hormone (24, 25). However, no difference between the activity of recombinant yhPTH-(1–84,Q26) and standard shPTH-(1–84) could be detected in three different biological assays: an adenylate cyclase assay, measurement of the ability to induce hypercalcemia in parathyroidectomized rats, and the mouse calvarial assay for bone resorption-stimulating activity. It is possible that the dibasic site in hPTH in fact functions as an easily attacked proteolytic site that destabilises the hormone and thus allows more rapid fluctuations in the hormone levels. A more degradation-resistant hPTH agonist could therefore be of potential importance when used as a medical drug due to different pharmacokinetics.

hPTH is one of the primary calcium-regulating hormones in the body and acts principally on kidney and bone cells, stimulating renal calcium reabsorption, phosphate excretion, and bone remodelling, respectively (26–28), resulting in anabolic as well as catabolic effects. Its overall physiological action is probably to generate a positive calcium balance and enhance bone formation. A fragment of hPTH together with 1,25-$(OH)_2$ vitamin $D_3$ have also been reported to induce bone formation in humans (29, 30), and one of the major areas of potential use of a recombinant hPTH is therefore in the treatment of postmenopausal osteoporosis. To evaluate such applications, sufficient supplies of recombinant hPTH or its agonists are essential.

*Acknowledgment*—We are grateful to Dr. Knut Sletten, Department of Biochemistry, University of Oslo, Norway, for doing the amino acid sequence and composition analysis.

[2] Portions of this paper (including "Materials and Methods," "Results," Figs. 1–6, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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**Supplemental material**
**to**

# Characterization of a K26Q site directed mutant of human parathyroid hormone expressed in yeast

**by**

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## MATERIALS AND METHODS

*Plasmids, strains and culture conditions.*

The *Saccharomyces cerevisiae* strain used for bPTH expression was BJ1991 (a, *trp1, ura3-52, leu2, prb1-1122, pep4-3* ). Yeast cells were transformed by the lithium method (31), and transformants grown at 30°C in YNBCC medium (6.67% yeast nitrogen base, 2% glucose, 1% casamino acids (Difco)). The puUXPTH-3 plasmid used as a reference for expression of authentic bPTH(1-84) in yeast, and the subclone M13PTH-3, have been described previously (13; US Patent application no.921686).

*In vitro mutagenesis of the α-factor bPTH fusion gene.*

The inappropriate processing of bPTH secreted from yeast (cleaved at the site: Arg25Lys26|Lys27 (12)), might be eliminated by a change from Lys26 to Gln26 to destroy the potential yscF processing site. To introduce this change, the subclone M13PTH-3 was transfected by *in vitro* mutagenesis using the "Mutagene™ *in vitro* mutagenesis kit" (Bio-Rad) based on the method of Kunkel *et al.*, (32) and the mutagenizing oligonucleotide: 5'-GGLTGCGTCAGAAGCTGC-3'. Positive plaques (containing M13PTH-Q26) were verified by DNA sequencing (33). The mutre expression cassette between a *BamHI* and a filled-in *EcoRI* site was isolated from M13PTH-Q26 and inserted between the *BamHI* and *PvuII* site of the yeast shuttle vector YEp24 (34). This expression plasmid was designated puUXPTH-Q26 and used to transform the yeast strain BJ1991.

*Detection of hPTH in culture media.*

Radioimmunoassay of bPTH in yeast culture media was performed as described (12,35). For electrophoretic analysis, yeast culture media were concentrated on S Sepharose FF as previously described (13), and separated on a 15% polyacrylamide gel in the presence of SDS (36), and silver stained with silver (37) or analyzed further by protein blotting using Immobilon PVDF Transfer Membranes (Millipore) and the buffers of Towbin *et al* (38). Reference hPTH(1-84) was purchased from Peninsula Laboratories (USA) and Sigma (USA). Protein blots were visualized as described (12).

*Purification of hPTH from yeast culture medium.*

The sterilized culture medium was concentrated on a 5 Sepharose Fast Flow column previously equilibrated with 0.3M glycine, pH 3.5. The medium was adjusted to pH 3.0 with 1 M HCl before loading. The column was then washed with 0.1M acetic acid (adjusted to pH 4.0) and eluted with 0.1M $Na_2HPO_4$, pH 6.5. hPTH contained with the $A_{280}$ peak. This step also resulted in a partial purification of the hormone. Final purification was obtained by a two-step reverse phase HPLC procedure. In the first step a Vydac protein/peptide C18 column (0.46x4.6 mm, purchased from "The separation group", Hesperia, CA,USA) was employed with a gradient from 40-60% eluent B (eluent A: 0.2% trifluoroacetic acid in milliQ water, and eluent B: 0.1% trifluoroacetic acid in 70% acetonitrile and 30% milliQ water). The flow rate was 1 ml/min. In the second step, a Waters Nova-Pak C18 column (15.0x0.8 cm) was used with isocratic elution using 47% eluent B. Both elution were monitored by UV at 220 nm.

*N-terminal sequence, amino acid composition and mass spectrometry*

Proteins to be sequenced were purified either by HPLC as described above or by SDS polyacrylamide gelelectrophoresis followed by blotting onto Immobilon PVDF membranes (39). Automated Edman degradation was performed on a 477A Protein Sequencer with on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA, USA). All reagents were obtained from Applied Biosystems. The amino acid composition was analyzed on a Biotronic ST6100 amino acid analyzer after hydrolysis of 30 µg of the bPTH peptide in 6M HCl (special purity), 10.05% thioglycol acid, for 24 hr at 110 °C.

The molecular mass of purified recombinant bPTH(1-84,Q26) was determined by $^{252}Cf$ plasma desorption mass spectrometry as described (18), and was kindly performed by Dr. Mats Karlsson, KabiVac AB, Stockholm.

*Adenylate cyclase assay for bPTH.*

The adenylate cyclase stimulating activity of the recombinant bPTH was assayed as previously described using UMR 106 osteosarcoma cell membranes (17,40,41). Synthetic hPTH(1-84) from Sigma was used as reference standard.

*Assay of hPTH-induced hypercalcemia in rats*

Male Wistar rats (150-200 g) were parathyroidectomized using electrocautery 18 hours prior to the start of the experiment. The animals were fasted overnight, and anesthetized the next day using Hypnorm Dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers Acetate, 4% bovine albumin, 25 Units heparin/ml. Five minutes after injection of 200 µl of the heparinized Ringers, a baseline blood sample was drawn (300 µl). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. PTH was then injected subcutaneously in the neck in a volume of 200 µl. Both hPTH(1-84) and hPTH(1-84,Q26) were dissolved in 100 µl 0.01N acetic acid. After dissolving, the solutions were brought up in 900 µl of Ringers Acetate containing 1% bovine albumin. Blood samples were drawn from the carotid artery at 1, 2, 3, 4, 5, and 6 hours after the injection of PTH. The rats were re-heparinized 5 minutes before drawing each blood sample using 200 µl of the heparinized Ringers solution. The concentrations of PTH used were determined by amino acid analysis. hPTH(1-84,Q26) was used at 3 pg/animal, and the Bachem reference PTH was used at 3.75 pg/animal (a higher numerical value used to compensate for a discrepancy between what was labelled on the Bachem vial (1 mg PTH), and what we determined by amino acid analysis (0.75 mg PTH)). The blood samples were centrifuged for 10 minutes, and the plasma was analyzed for calcium using a Cobas Autoanalyzer (Cobas Bio, Roche Diagnostics).

*Bone resorption-stimulating activity*

Bone resorption-stimulating activity was measured using neonatal mouse calvaria as previously described (42,43). The medium was Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated (56-60°C for 30 min) horse serum. Incubation of calvaria from 4- to 5-day-old mice was performed at 37°C in a roller drum apparatus under an atmosphere of 50% $O_2$, 5% $CO_2$, and 45% $N_2$. Calvarias were preincubated for 24 hours before experimental treatment was begun. The medium was then changed, and fresh control medium or medium containing standard synthetic PTH (shPTH(1-84)), yeast derived hormone (yhPTH(1-84)), or yeast derived mutant hormone (yhPTH(1-84,Q26)) was added. Bone resorption was determined by measuring the accumulation of $^{45}Ca^{2+}$ in the culture medium during the 72-hour treatment period. The concentration of total calcium in the bone culture medium was measured in 400 µl samples by a calcium-selective electrode using a Nova 7+7 automatic calcium analyzer (NOVA Biomedical, Waltham, MA, USA). Results in each experiment were subjected to an analysis of variance, and the SE values were calculated from the residual error term of that analysis. Experiments were pooled when the variances were homogeneous. The shPTH(1-84) standard was from Bachem (Lot no. 9705589).

## RESULTS

*Expression and secretion of hPTH(1-84,Q26) in yeast.*

To prevent the internal processing of bPTH occuring during secretion in yeast, a point mutation was introduced in codon 26 of the bPTH gene changing lysine-26 to glutamine in the peptide. The mutated gene was expressed in *Saccharomyces cerevisiae* after fusion to the α-factor prepro-leader sequence and promoter. The products secreted to the growth medium were analyzed by SDS-PAGE (Fig.1). In the non-mutated control (lane 1) the strongest band had a molecular mass well below the standard bPTH, and subsequencing showed that it corresponded to the hormone fragment bPTH(17-84) (12). After mutation (lane 3), this band was absent, showing that the cleavage between amino acid 26 and 27 was eliminated as a result of the introduced mutation. The major product was a polypeptide that migrated close to the full length bPTH standard, slightly faster in an anionic gel system (Fig.1) and slightly slower in a cationic gel system (not shown), in accord with the single charge difference between the mutated and the wild type protein. In addition to the main product, several other bands were present of apparently higher molecular mass which appear to be O-glycosylated forms of yhPTH(1-84,Q26) (unpublished experiments). The yhPTH(1-84,Q26) product also migrated close to the bPTH standard when analyzed by two dimensional gel electrophoresis (first dimension acetic acid/urea gel, second dimension SDS polyacrylamide gel, not shown) and, by protein blotting, it was found to react with two bPTH specific antibodies against the 1-34 amino acid and 44-68 amino acid respectively (not shown), supporting that we are dealing with a full length hormone.

*Purification*

To purify yhPTH(1-84,Q26), growth medium was first concentrated using a S Sepharose FF column (yielding 70% pure yhPTH(1-84,Q26)) (Fig.1). The bPTH peak from the first HPLC step contained predominantly yhPTH(1-84,Q26), but also some glycosylated hormone with reduced migration in SDS PAGE (Fig.2A). After a second isocratic HPLC chromatography, only a single band was seen after SDS PAGE (Fig.2B). When the yield from shake-flask cultures of pure yhPTH(1-84,Q26) was compared with that of yhPTH(1-84), 5-10 fold higher yields were obtained, consistent with our previous estimate of the fraction of full length hormone (up to 20%) produced with an expression plasmid encoding non-mutated bPTH(1-84) (12).

*Biochemical characterization of yhPTH(1-84,Q26)*

The nature of the yhPTH(1-84,Q26) product was verified by N-terminal amino acid sequencing showing correct amino-terminal sequence (Table 1). In particular, the expected change from Lys to Gln in position 26 was confirmed. The amino acid composition of the purified yhPTH(1-84,Q26) is shown in Table 2. Except for one Trp that is not revealed under the conditions used, the expected composition and total number of amino acid residues were found. Therefore, no C-terminal degradation had occurred. To substantiate that the purified yhPTH(1-84,Q26) representing the intact hormone, mass spectrometry was performed giving a plasma desorption mass spectrum as shown in Fig.3. A molecular mass of 9432±2 daltons could be calculated from the singly-charged and double-charged molecular ions present in the spectrum. This value corresponds well with the theoretical molecular mass of hPTH(1-84,Q26) calculated from the amino acid composition, of 9418.

*Biological characterization*

The biological activity of the yhPTH(1-84,Q26) was tested by three different assays. The purified yhPTH(1-84,Q26) was first analyzed for its ability to stimulate the adenylate cyclase activity in rat UMR 106 osteosarcoma cells (40,41) (Fig.4). The stimulation curve coincides with that of purified non-mutated yhPTH(1-84). In this assay, no significant differences in biological activity was detected between the native hormone and the degradation-resistant mutated hormone.

Secondly, the purified yhPTH(1-84,Q26) and a standard shPTH(1-84) were assayed for the ability to induce hypercalcemia in parathyroidectomized rats (Fig.5). The yhPTH(1-84,Q26) had an *in vivo* biological activity stimulatory effect comparable to or slightly above that of the synthetic bPTH control.

Finally, the yhPTH(1-84,Q26) was tested for direct activity on bone by assaying its bone resorption-stimulating activity using neonatal mouse calvaria. yhPTH(1-84,Q26) stimulated bone resorption to give with a potency similar to that of shPTH(1-84) and yhPTH(1-84) (Fig.6). A time-course experiment similarly showed that yhPTH(1-84,Q26) had the same bone resorption-stimulating activity as the non-mutated hormone (not shown). Thus, we have found no indication of reduced biological activity resulting from the introduced mutation.

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# High-level production of human parathyroid hormone in *Bombyx mori* larvae and BmN cells using recombinant baculovirus

(PTH; cDNA; silkworm; cells; osteoblast function tests)

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SUMMARY

A full-length cDNA encoding human parathyroid hormone (hPTH) containing the prepro region was cloned into *Bombyx mori* baculovirus under the control of the polyhedrin promoter and polyadenylation sequences. After transfection and generation of the recombinant baculovirus, hPTH production was examined in silkworm larvae and BmN cell cultures. The larvae synthesized and efficiently secreted the correctly processed and authentic hPTH (9.4 kDa) with no sign of internal degradation. In BmN cells, the major secreted form was the correctly sized protein, but small amounts of degraded hPTH could also be detected in the medium by immunoblotting. Unlike the situation in larvae, prepro-hPTH could also be demonstrated intracellularly in BmN cells. The concentration of hPTH in the larval hemolymph was about 70 mg/l, as compared to approx. 55 μg/l in the medium per $7.5 \times 10^6$ cells. Recombinant hPTH (re-hPTH) from the hemolymph was purified by reverse-phase HPLC and subjected to chemical and biological analyses. The authenticity of the purified re-hPTH was confirmed by N-terminal sequencing, amino acid composition and a mass of 9425 Da, close to the theoretical value. The hormone showed high-affinity receptor binding and full biological potency in increasing cellular cAMP.

## INTRODUCTION

Human parathyroid hormone (hPTH) is synthesized in the parathyroid glands as a prepro-hormone consisting of 115 aa. During processing, the pre and pro parts of the hormone are sequentially cleaved off resulting in the formation of the mature 84-aa hormone (Cohn and MacGregor, 1981). hPTH (1–84) is secreted in response to a lowering of serum $Ca^{2+}$ ions, and its physiological function is to elevate serum $Ca^{2+}$ and to maintain the calcium and phosphate homoeostasis (Potts et al., 1982; Reeve et al., 1980). Prolonged and intermittent administration of low to medium doses of biologically active hPTH fragment has been shown to vigorously stimulate bone formation in animals and patients with osteoporosis (Reeve et al., 1980; 1991; Bradbeer et al., 1992).

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Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); ACN, acetonitrile; *B.*, *Bombyx*; *Bm*, *B. mori*; BmN, *Bm* ovarian cell line; BmNPV, *Bm* nuclear polyhedrosis virus; bp, base pair(s); Bv, baculovirus; cAMP, cyclic AMP; cDNA, DNA complementary to RNA; cpm, count(s) per minute; DEPC, diethyl pyrocarbonate; HPLC, high-performance liquid chromatography; hPTH, human PTH; *hPTH*, gene (DNA) encoding hPTH; kb, kilobase(s) or 1000 bp; MS, mass spectrometry; LLC-PK$_1$, porcine renal epithelial cell line; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; pfu, plaque-forming unit(s); PPG, polypropylene glycol(s); PTH, parathyroid hormone; PTHrP, PTH-related protein; re-, recombinant; *S.*, *Saccharomyces*; SDS, sodium dodecyl sulfate; TFA, trifluoro acetic acid; *UTR*, untranslated region(s); wt, wild type.

Considering the potential pharmaceutical importance of the hormone in treatment of bone metabolic disorders, attempts have been made to produce the hormone employing different expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae* and mammalian cells (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Rokkones et al., 1994). The existence of internal protease sensitive domains has made hPTH susceptible to degradation and inactivation (Høgset et al., 1990; Gabrielsen et al., 1990). Thus, an optimalized system for expression of h*PTH* demands a correct processing without aberrant cleavage so that a high production efficiency can be obtained.

In this paper we compare the expression of h*PTH* in BmN cells and *Bombyx mori* (*Bm*) larvae using the *Bm* baculovirus with the polyhedrin promoter and regulatory sequences (Maeda, 1989a,b). The results demonstrate that the cells and the larvae are fully able to recognize the human signal and pro-part of hPTH.

## RESULTS AND DISCUSSION

### (a) Construction of recombinant virus for h*PTH* expression

Strategies followed for the cloning of full length h*PTH* cDNA (from pPPTH7) including its prepro part into the vector pBm030 is shown in the Fig. 1 and explained in legend. The re-vector pBmPTH84 harbours the full-length h*PTH* cDNA, including the human signal(pre) sequence and its pro part and is controlled by virus regulatory elements. Cotransfection of BmN cells in culture with the plasmid pBmPTH84 DNA and wt viral DNA (BmNPV) resulted in the formation of polyhedrin-negative re-plaques. Upstream and downstream from the cloned h*PTH* cDNA, about 3 kb viral flanking sequences are present, and during cotransfection, these flanking regions will facilitate homologous recombination so that the polyhedrin gene of the wt virus is replaced with the h*PTH* cDNA. After identification and isolation of re-viral plaques they were purified as described in Methods in the legend to Fig. 2. The re-virus were screened and those giving highest expression of h*PTH* were chosen for further experiments.

### (b) Production and secretion of hPTH into larval hemolymph

Hemolymph samples from larvae infected with re-virus and collected after 24, 48 and 72 h, showed a time-dependent increase in two peptides (9.4 kDa and 14.3 kDa) which immunoreacted with hPTH antiserum, while hemolymph from wt virus-infected larvae was negative (Fig. 2A, lanes 2, 3 and 4 versus 1). When standard hPTH(1–84) was loaded on the gel, it appeared as a 9.4-kDa form as expected (Fig. 2A, lane 6). However, when the same standard was mixed with the control-hemolymph, the same two different immunoreactive peptides appeared (9.4 kDa and 14.3 kDa) (Fig. 2A, lane 5). Thus, the 14.3-kDa band appeared to be a hPTH-binding protein as also confirmed in subsequent analysis. hPTH production increased during this period and the highest level was obtained after 72 h, whereafter the larvae succumbed to an infection. A semiquantitative estimation of hPTH in hemolymph collected the 3rd day of infection was carried out. Comparing the intensities of immunoreactivity to the different amounts of known hPTH standards as shown in Fig. 2B when different amounts of hemolymph sample was analyzed, it was estimated that 4 µl contained 0.25–0.5 µg hPTH (Fig. 2B, lanes 5, 6 and 7 versus lanes 2, 3 and 4). The non saturable binding properties of the 14.3-kDa band was verified by addition

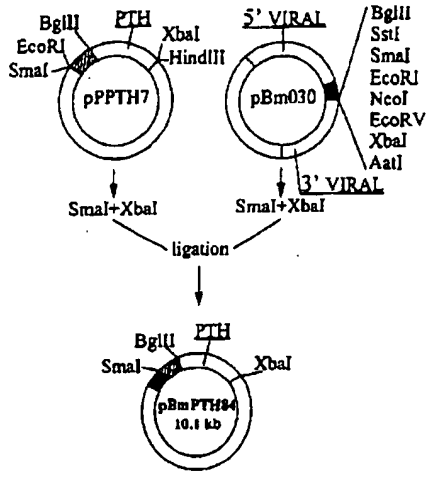


Fig. 1. Construction of baculovirus vector pBmPTH84. The entire h*PTH* cDNA signal and coding sequences were excised from the plasmid pPPTH7 by *SmaI* + *XbaI* digestion and ligated between the *SmaI* and *XbaI* sites of the vector pBm030 (Maeda, 1989b). In the resulting re-vector pBmPTH84 lacking the polyhedrin-encoding gene, the h*PTH* coding sequence is under the control of the polyhedrin promoter, transcriptional termination and polyadenylation sequences, but employing hPTH signal sequence. **Methods**: Plasmid DNA manipulations were performed essentially as described by Sambrook et al. (1989). A h*PTH* cDNA 413-bp fragment from plasmid pPPTH7 was ligated into vector pBm030, after cleavage with *SmaI* + *XbaI*, and transformed into the *E. coli* DH5α. The clones that contained the h*PTH* cDNA insert were identified and the re-vector named pBmPTH84. The junctions of h*PTH* cDNA and the transfer vector were confirmed by sequencing. Restriction enzymes and other DNA metabolizing enzymes were obtained from New England Biolabs. A anti-rabbit-[$^{125}$I]IgG was from Amersham. Synthetic hPTH (1–84) from Bachem was used as standard. All the other chemicals used were from Sigma.

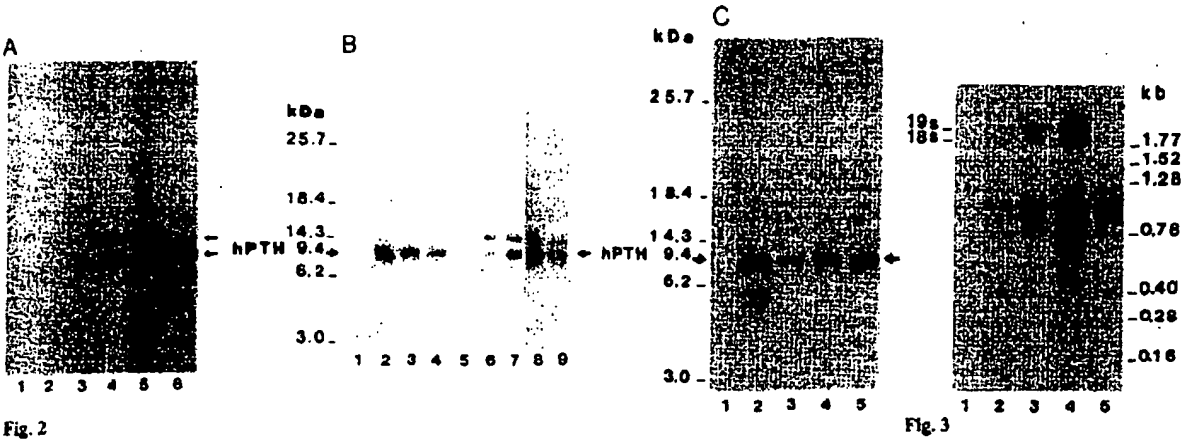


Fig. 2

Fig. 3

Fig. 2. The re-h*PTH* expression. (A) Time-course study of h*PTH* expression in hemolymph from infected larvae collected after 24, 48 and 72 h of infection with re-baculovirus (lanes 2, 3 and 4) compared to control represented by 72 h of wt infection (lane 1) using Western blot technique and mid-region specific anti-hPTH antibody. 3 µl per lane using 0.1% SDS-15% PAGE. Lane 5: hemolymph from wt infected larvae added 0.25 µg hPTH(1–84) standard. Lane 6: 0.25 µg hPTH(1–84) standard. (B) Semiquantitative estimation of hPTH produced in hemolymph 3 days after infection with wt (lane 1 (4 µl)) and re-baculovirus (lanes 5 (1 µl), 6 (2 µl) and 7 (4 µl)) and subjected to 0.1% SDS-15% PAGE, followed by immunoblot analysis. Lanes 2, 3 and 4: hPTH(1–84) marker (Bachem), 0.75 µg, 0.5 µg and 0.25 µg, respectively. Lanes 8 and 9: 4 µl of hemolymph from wt infected larvae added 0.5 µg and 0.25 µg hPTH(1–84) marker, respectively. (C) hPTH in BmN cell cultures 4 days after infection with re-virus (lane 2) and wt virus (lane 1). 5 ml of culture medium partly purified on a Sep-Pak column freeze-dried and analyzed by SDS–PAGE as in panel B. Lanes 3, 4 and 5: 0.05 µg, 0.1 µg and 0.075 µg, respectively, of hPTH(1–84) standard. **Methods**: *Bm larva and BmN cell culture*: The silkworm *Bm* larvae (TW × NB4D2) were fed ad libitum on fresh mulberry leaves and reared in the laboratory following the method of Krishnaswamy et al. (1973). BmN cells were grown in TC-100 medium containing 10% fetal calf serum and 50 µg gentamycin per ml at 27°C (Maeda, 1989a,b). *Transfection and isolation of re-virus*: Re-vector pBmPTH84 was amplified and purified. Subconfluent monolayers of BmN cells were co-transfected with purified infectious *Bm* wt baculovirus (BmNPV) DNA and the re-vector pBmPTH84. Homologous recombination between the plasmid DNA and wt viral DNA occurred in the Ca-phosphate mediated cotransfected cells as tested after 5 days by plaque assay, and the polyhedrin-negative plaques were screened for hPTH production in the BmN cells and *Bm* larvae. *Collection of larval hemolymph, fatbody, BmN cell culture medium and cell lysate*: Early fifth instar (24 h old) *Bm* larvae were needle inoculated with 50 µl of recombinant viral solution ($3 \times 10^5$ pfu) into the body cavity using wt virus and saline injections as controls. Hemolymph was collected and treated as described (Maeda, 1989a). BmN cells ($7.5 \times 10^6$ cells) were seeded in a tissue culture flask and after overnight incubation, the cells were infected by re-virus or wt virus (10 pfu/cell) separately. After 4 days of infection the medium was collected, the samples centrifuged at 1400 rpm for 5 min and the medium and cell pellet stored separately. All the samples were stored at −70°C till further analysis. *Protein determination*: Protein in hemolymph was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. *PAGE and immunoblotting*: 0.1% SDS-15% PAGE was performed under reducing conditions (Laemmli, 1970) and samples were solubilized as previously described (Towbin et al., 1979; Gabrielsen et al., 1990). *Semiquantitative protein determination of hPTH*: Semiquantitative measurements of hPTH using light scanning were performed on X-ray films developed after Western immunoblots using Bio Image system, Millipore.

Fig. 3. mRNA was prepared from fatbody cells and analyzed on agarose gels followed by Northern blot and hybridization to a h*PTH* cDNA fragment. Lanes: 1, 1.3 µg mRNA from wt-infected larvae (control) 72 h post-infection; 2, 3 and 4, 1.3 µg mRNA from re-virus-infected larvae 24, 48 and 72 h after infection; 5, 1 µg of PTH-mRNA isolated from human parathyroid adenomas. Ribosomal RNAs corresponding to 18S and 19S, respectively, are indicated. **Methods**: Total RNA was extracted from larval fatbody (wt virus infected and re-virus infected) as described (Glišin et al., 1974). Poly(A)$^+$RNA was selected from identical amounts of total RNA from all the extractions using magnetic oligo(dT) Dynabeads (Dynal A.S. Norway). For time-course response, RNA was extracted at every 24 h post infection for a period of 3 days. The poly(A)$^+$RNA samples were subjected to electrophoresis on a 1.5% agarose gel containing 6% (v/v) formaldehyde in 20 mM Na-phosphate pH 7.0 buffer. The RNA was subsequently transferred to a nylon membrane by passive diffusion and immobilized by UV light (2 min) and baked at 80°C for 1 h. The h*PTH* cDNA *Xba*I-*Eco*RI fragment was used for probe, and filters were subjected for hybridization at 42°C following standard procedures (Sambrook et al., 1989).

of 0.5 µg and 0.25 µg of hPTH, respectively, to the wt hemolymph (Fig. 2B, lanes 8 and 9) which by itself contained non-detectable immunoreactivity (Fig. 2B, lane 1).

### (c) Production and secretion of hPTH by BmN cells into culture medium

BmN cells infected with re-virus also produced and secreted hPTH into the medium. Two secreted proteins were detected on immunoblots of SDS-PAGE using mid-region specific anti-h*PTH* antiserum (Fig. 2C, lane 2). One comigrated with hPTH(1–84) standard (9.4 kDa) and the other and somewhat diffuse 5.5-kDa immunoreactive band probably represented proteolytic degradation products. The amount of hPTH was estimated to be about 0.3–0.5 µg from the flask with an initial concentration of $7.5 \times 10^6$ cells as determined by light scanning of the X-ray films developed after various times and compared to hPTH standards (lanes 3, 4 and 5). No immuno-

reactivity was found in medium from wt virus-infected cells (Fig. 2C, lane 1).

**(d) The presence of h*PTH* mRNA**

The presence of h*PTH* mRNA in fatbody cells was also studied 24, 48 and 72 h after infection and analyzed on agarose gels followed by Northern-blot and hybridization to a *PTH*cDNA *Xba*I-*Eco*RI fragment (Fig. 1) as probe. A time-dependent increase (about 100-fold) in transcripts corresponding in size to *PTH*mRNA prepared from human parathyroid adenomas was observed (Fig. 3, lanes 2, 3 and 4 versus lane 5). In addition, two higher $M_r$ transcripts appeared of sizes equal to 18S and 19S rRNAs.

The presence of three mRNA species in the fat body may indicate heterogeneity within the non-translated regions since only one peptide form was demonstrated. They cannot be due to non-specific hybridization to remaining ribosomal RNA, since wt RNA gave no signal (Fig. 3, lane 1); in addition, a time-dependent increase was also observed.

**(e) Intracellular hPTH in larvae and in cultured cells**

Intracellular proteins from larval fatbody and BmN cells infected with re-virus were examined using immunoblots and compared to wt virus-infected controls. No hPTH immunoreactivity was detected in the larval fatbody cells while BmN cells showed two dominant hPTH immunoreactive bands. The major one of 13.5 kDa was similar to unprocessed prepro-hPTH while the 16-kDa protein could represent a modified variant or protein bound form of prepro-hPTH (data not shown).

**(f) Quantitative measurements of hPTH by two-site chemiluminometric (sandwich) immunoassay**

hPTH(1–84) in the hemolymph and culture medium was assayed using chemiluminometric immunoassay according to the manufacturer (Magic Lite, Ciba Corning, Germany). In hemolymph collected three days after coelomic infection, the hPTH concentrations were 0.05–0.1 g/l, while the total protein concentration was 63 g/l. In BmN cell culture medium at day 4 postinfection, the maximal hPTH concentrations were 40–70 μg/l per $10^6$ cells.

**(g) Reverse-phase HPLC-purification of hPTH and assessment of the chemical purity and authenticity**

The re-hPTH was extracted from hemolymph and further purified as described in Methods to Fig. 4. The HPLC purification profiles are shown in Fig. 4 (A, B and C). hPTH from the last HPLC-step (Fig. 4C) was analyzed further on SDS-PAGE (Fig. 4D and E). The results from the gel analyses, including silver-staining (Fig. 4D) and immunoblot analysis (Fig. 4E) showed only one band with a mobility equal to standard hPTH and a purity equal to or better than the hPTH(1–84) Bachem standard (Fig. 4D and 4E, lanes 1 and 3 compared to lanes 2 and 4).

The purified PTH was also subjected to aa composition analysis and N-terminal sequencing which were consistent with the theoretical prediction (data not shown). Mass spectrometry was performed with a spectrum as shown in Fig. 5A and an $M_r$ of 9425 was obtained from the single-charged molecular ions corresponding well

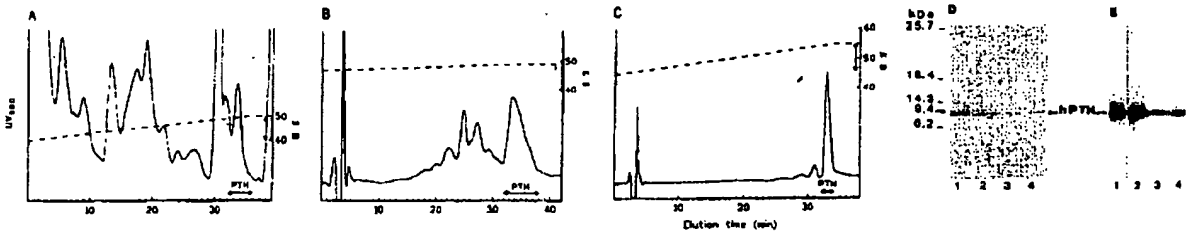


Fig. 4. Separation of re-hPTH(1–84) by reverse-phase HPLC from hemolymph of infected silkworms (A, B and C) and characterization by SDS-PAGE (D and E). (A) Preparative scale chromatography was performed on a Pharmacia SuperPac Pep-S $C_{18}/C_2$ column (22.5 × 250 mm) as previously described by Olstad et al. (1992) with small modifications. Eluant A, 0.115% TFA in MilliQ water; eluant B, 0.085% TFA in 70% aqueous acetonitrile (ACN) (10 ml/min). (B) Pooled fractions from A) containing hPTH were analyzed on an analytic Pharmacia SuperPac Pep-S $C_{18}/C_2$ column (4.0 × 250 mm) (linear gradient of 47–49% eluant B run for 35 min). Eluant A and eluant B were the same as in A. The flow rate was 1.0 ml/min. (C) Pooled fractions from B) containing hPTH were further purified on Pharmacia SuperPac Pep-S $C_{18}/C_2$ column (4.0 × 250 mm). A linear gradient of 45–55% eluant B was run for 35 min. Eluant A, 0.55% TFA in MilliQ water; eluant B, 0.45% TFA in 70% aqueous ACN. The flow rate was 1.0 ml/min. (D and E) 0.1% SDS-15% PAGE analysis of HPLC purified hPTH from hemolymph (Fig. 3C, fractions 33 and 34) and compared to hPTH(1–84) standard from Bachem. (D) Silver-staining. (E) Immunoblot analysis using the mid-region specific anti-hPTH antiserum. Lanes 1 and 3: 1.0 μg and 0.2 μg of hPTH from hemolymph. Lanes 2 and 4: 1.0 μg and 0.2 μg hPTH(1–84) standard (Bachem). **Methods:** *Purification of hPTH from the medium*: hPTH was concentrated and partly purified as described previously (Olstad et al. 1992) and after freeze-drying, the samples were dissolved in sample-buffer for SDS-PAGE analyses (Laemmli, 1970). *Reverse-phase HPLC*: Preparative and analytical scale chromatography was performed as described previously on SuperPac Pep-S $C_{18}/C_2$ column (22.5 × 250 mm/4.0 × 250 mm) (Reppe et al., 1991; Olstad et al., 1992). For silver staining of the gel, the procedure for the Sigma silver stain kit was followed.

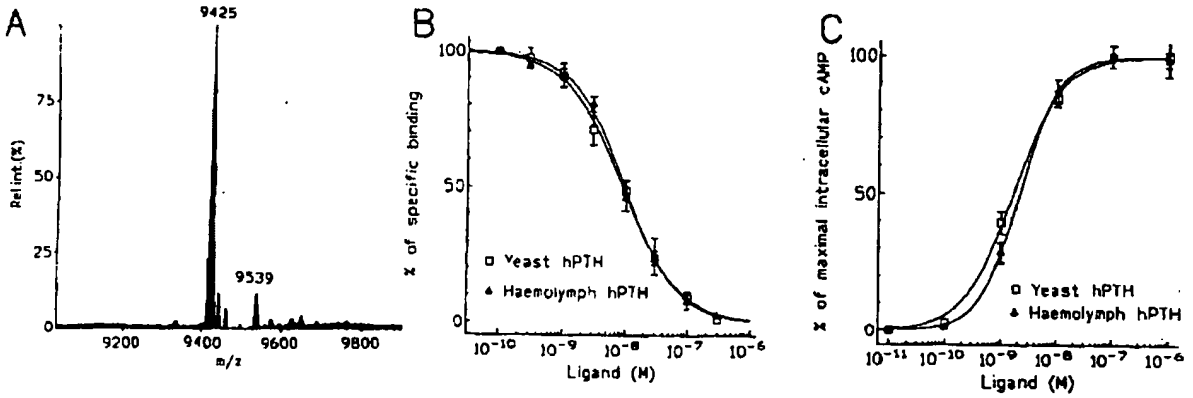


Fig. 5. Characterization of re-hPTH by mass-spectroscopy (A), radioreceptor binding (B) and intracellular cAMP stimulation (C). (A) Mass-spectroscopic analysis of recombinant hPTH(1–84). The dominant single peak represents a molecular hPTH mass of 9425 Da corresponding to the theoretical value of 9424.7 Da. (B) Inhibition of radiolabeled [Tyr$^{36}$]chicken-PTH-related protein(1–36)amide by different hPTHs. The re-hPTH(1–84) produced in infected silkworms and recombinant hPTH(1–84) (yeast hPTH) produced in *Saccharomyces cerevisiae* were tested in a radioreceptor assay using LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean ± SEM of three independent experiments each performed in triplicate. (C) Stimulation of cellular cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor and stimulated (15 min, 37°C) with re-hPTH(1–84) produced in silkworms and re-hPTH(1–84) (yeast hPTH) produced in *Saccharomyces cerevisiae* is shown. The data represent the mean ± SEM of three independent experiments each performed in duplicate. **Methods**: *Mass spectrometry analysis* was performed using a API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada) and carried out as described (Covey et al., 1988). *Radioreceptor assay*: LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993), were plated in 24-well plates (50 000 cells/well) and grown for 2 days before incubation with $^{125}$I-labelled [Tyr$^{36}$]chicken-PTHrP(1–36)NH$_2$ (100 000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris·HCl, pH 7.7/100 mM NaCl/5 mM KCl/2 mM $CaCl_2$/5% heat-inactivated horse serum/0.5% heat-inactivated fetal calf serum) as described (Jöppner et al., 1988). The competing ligands were recombinant hPTH(1–84) expressed in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and recombinant hPTH(1–84) purified from hemolymph of infected silkworms. Techniques used for radioiodination of PTHrP analog were previously reported. *Intracellular cAMP measurements*. Measurements of intracellular cAMP in LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993) using Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min, then washed and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 M HCl. The stimulating ligands were re-hPTH(1–84) produced in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and re-hPTH(1–84) produced in *Bm* hemolymph.

with the theoretical $M_r$ of 9424.7 for hPTH as calculated from the aa composition.

### (h) Radioreceptor binding studies and intracellular cAMP measurements

Binding properties of two different re-hPTH forms from yeast and silkworm are shown in terms of displacement curves using the $^{125}$I-labeled [Tyr$^{36}$]chicken-PTHrP(1–36)NH$_2$ as radioligand and LLC-PK$_1$ cells permanently transfected with the rat PTH/PTHrP receptor. Both hPTH forms representing the authentic hormones, showed equal receptor binding affinities (Fig. 5B) (calculated $K_d = 8.8 \pm 1.2 \times 10^{-9}$ M) and identical abilities to stimulate intracellular cAMP accumulation in the same cells with half maximal response obtained at $2.0 \times 10^{-9}$ M (Fig. 5C).

### (i) Concluding remarks and comparison of h*PTH* expression between various host systems

The quantitative result of re-hPTH production in BmN ovarian cell culture was much less than that in the hemolymph even corrected for the 30%–50% loss during medium concentration and Sep-Pak column chromatography (see Methods in the legend to Fig. 4). The circulatory system of silkworm larvae opens into the coelomic cavity which is totally bathed in the hemolymph and retains the secretory proteins (Shigematsu, 1958).

hPTH could not be detected in the intracellular fractions of larval fatbody while the polypeptide and the assumed unprocessed forms were present in BmN cells. Ovarian cells are normally not designed for secretion of proteins, but rather for absorption, and this may be a reason for the low level of hPTH produced. The hormone and its mRNA were expressed in a parallel and a time-dependent fashion. The hPTH produced in the silkworm larvae was authentic as judged by N-terminal sequence, total aa composition and mass spectrometry. Also its receptor binding affinity and ability to activate the main second messenger system were identical to the yeast re-hPTH which previously was shown to have full biological activity in several target cell systems (Reppe et al., 1991).

The amount of hormone produced in the larval hemolymph represented 70 mg/l. This level was many times

higher than that reported for yeast (Gabrielsen et al., 1990; Reppe et al., 1991) and for *E. coli* (Høgset et al., 1990) secreted hPTH. The re-hPTH produced as an intracellular fusion protein with *S. aureus* protein A, showed a production after purification of 50–80 mg/l culture (Forsberg et al., 1991).

The production yield of hPTH in the silkworm larvae also compared well to ZZ-cecropin A fusion protein production in *Trichoplusia ni* larvae using *Autographa californica* baculovirus (Andersons et al., 1991). Production of human α-interferon in silkworm using *Bm* baculovirus, amounted to 30 mg/l hemolymph after purifying 10 ml of hemolymph by affinity column chromatography (Maeda et al., 1985). However, they did not report the concentration of α-interferon in the hemolymph prior to purification.

Expression of h*PTH* in microbiological systems has met with two problems related to incorrect N-terminal cleavage and aberrant intracellular processing (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Reppe et al., 1991; Forsberg et al., 1991; Rokkones et al., 1994). The major cleavage sites were after $Lys^{26}$ in *S. cerevisiae* and after $Val^{21}$ in *E. coli* (Rokkones et al., 1994). In mammalian cells, in contrast (mouse mammary tumor cell, i.e., C1271 cells, and Chinese hamster lung cells, i.e., DON cells) the entire h*PTH* cDNA including the prepro part gave rise to only the intact form (Rokkones et al., 1994). We demonstrate that the silkworm larvae in fact resembles the mammalian system cleaving the hPTH signal sequence correctly and that the human signal is also able to promote an efficient secretion of the intact hormone. However, the hPTH produced binds to a natural protein in the hemolymph and gives rise to a 14.3-kDa protein in addition to the expected 9.4-kDa form. The N-terminal sequence of the purified '14.3-kDa protein' was identical to hPTH(1–84) (data not shown). Standard hPTH also showed the same two bands when added to the hemolymph (Fig. 2A and B). Moreover, in buffer containing urea, the mobility of the 14.3-kDa form was normalized (data not shown) and also the 'acid treatment' occurring during HPLC purification released the peptide. Thus, the '14.3-kDa protein band' represents a hPTH-binding protein of unknown nature.

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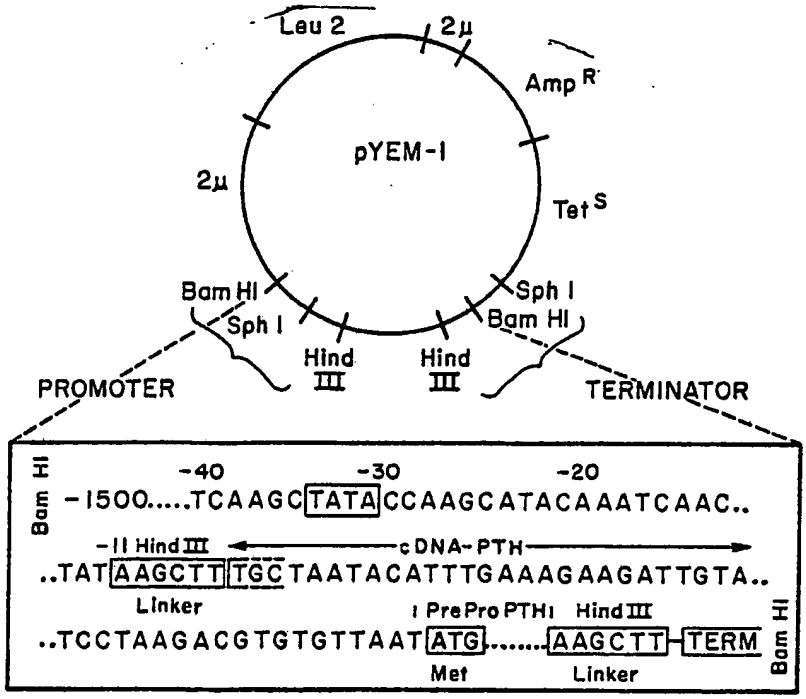
(54) Title: PRODUCTION OF MATURE PROTEINS IN TRANSFORMED YEAST

(57) Abstract

A method for producing a mature protein in yeast transformed to express a corresponding precursor, wherein the mature protein sequence is contained in the precursor and is flanked proximally or both proximally and distally by a pair or triplet of basic amino acid residues. The method comprises proteolytic processing by an endopeptidase and exopeptidase present in the yeast. Yeast transformed by a plasmid containing a cDNA sequence encoding bovine preproparathyroid hormone is also disclosed.



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# PRODUCTION OF MATURE PROTEINS IN TRANSFORMED YEAST

## BACKGROUND OF THE INVENTION

1. Field of the Invention.

This invention relates to a method for producing a mature protein in transformed yeast and further relates to Saccharomyces cerevisiae transformed by a plasmid containing a preproparathyroid hormone cDNA insert.

2. Description of the Prior Art.

Recombinant DNA technology now makes it possible to isolate specific genes or portions thereof from higher organisms, such as man and other animals, and to transfer the genes or fragments to a microorganism species, such as E. coli or yeast. The transferred gene is replicated and propagated as the transformed microorganism may become endowed with the capacity to make whatever protein the gene or fragment encodes, whether it be an enzyme, a hormone, an antigen or an antibody, or a portion thereof. The microorganism passes on this capability to its progeny, so that in effect, the transfer results in a new strain, having the described capability.

Recombinant DNA conventionally utilizes transfer vectors. A transfer vector is a DNA molecule which contains genetic information which insures its own replication when transferred to a host microorganism strain. Plasmids are an example of a transfer vector commonly used in genetics. Although plasmids have been used as the

transfer vectors for the work described herein, it will be understood that other types of transfer vectors may be employed. Plasmid is the term applied to any autonomously replicating DNA unit which might be found in a microbial cell, other than the genome of the host cell itself. A plasmid is not usually genetically linked to the chromosome of the host cell. Plasmid DNA exists as doublestranded ring structures generally on the order of a few million daltons molecular weight, although some are greater than $10^8$ daltons in molecular weight. They usually represent only a small percent of the total DNA of the cell. Transfer vector DNA is usually separable from host cell DNA by virtue of the great difference in size between them. Transfer vectors carry genetic information enabling them to replicate within the host cell.

Plasmid DNA exists as a closed ring. However, by appropriate techniques, the ring may be opened, a fragment of heterologous DNA inserted, and the ring reclosed, forming an enlarged molecule containing the inserted DNA segment.

Transfer is accomplished by a process known as transformation. During transformation, host cells mixed with plasmid DNA incorporate entire plasmid molecules into the cells. Once a cell has incorporated a plasmid, the latter is replicated within the cell and the plasmid replicas are distributed to the progeny cells when the cell divides.

Genetic information contained in the nucleotide sequence of the plasmid DNA, including heterologous DNA inserted into the plasmid, can in principle be expressed in the host cell. The inserted heterologous DNA typically representing

a single gene, is expressed when the protein product coded by the gene is synthesized by the organism.

Once a gene has been isolated, purified and inserted into a plasmid or other vector, the availability of the gene in substantial quantity is assured. After transfer of the vector into a suitable microorganism, the gene replicates as the microorganism proliferates. The vector containing the gene is easily purified from cultures of the host microorganism by known techniques and separable from the vector by restriction endonuclease cleavage followed by gel electrophoresis. The protein product expressed by the heterologous gene can also be recovered in substantial quantities from cultures of the host microorganism by harvesting the culture and retrieving the protein product contained in the harvested cells. (For futher detail of recombinant DNA technology, and an explicit exposition of the utility of producing proteins such as hormones, etc., by recombinant DNA technology, see U.S. Patent No. 4,237,224, issued December 2, 1980 to Cohen et al., and U.S. Patent No. 4,322,499, issued March 30, 1982 to Baxter et al. Patents and articles cited herein are incorporated by reference wherever such citations occur and shall be considered incorporated in their entirety as if set forth in full).

Recombinant DNA thus holds great promise for economically producing substantial quantities of useful proteins that are difficult or costly to isolate in such quantitites from mammalian tissue. A major and nearly universal problem in producing useful proteins, however, is the construction of the actual genetic material to be inserted into the transfer vector.

Conventional means provide for enzymatically preparing desired genetic material by reverse transcription. Mature messenger RNA (mRNA), which is chemically similar to DNA and retains most of the information coded in DNA, can be extracted from tissue in which the desired gene is active. mRNA is separated from other RNA material in the tissue and complementary DNA (cDNA) is produced by the enzyme reverse transcriptase, and at times polymerase 1 for the synthesis of the second strand. This cDNA, a complementary copy of mRNA and similarly containing the information coded in RNA, is often further altered in known ways to be suitable for insertion into a plasmid vector. (See W. Mahoney & S. Henikoff, Univ. of Washington Medicine, Vol. 8, No. 4, pp. 6-14 (Winter, 1981)).

cDNA enzymatically prepared by reverse transcription has the potential to express a protein chain identical to the protein expressed by tissue from which the mRNA was extracted. This alone is not sufficient, however, for the expression of desired mature animal proteins because many animal proteins, represented by such diverse classes as hormones, binding proteins, enzymes, antibodies, and collagen, are produced in nature in the form of larger precursors that are subsequently modified by cleavage to smaller bioactive forms commonly designated mature proteins. Thus, expression of cDNA synthesized by reverse transcription only has the potential to express the precursor of the mature protein product.

It has been known for several years that bacteria such as E. coli can remove the "pre" portion of its own secreated proteins. Examples include the processing of pre-ribose binding

protein, pre-galactose binding protein and pre-arabinose binding protein. (L. Randall, et al., Eur. J. Biochem., Vol. 92, pp. 411-415 (1978); L. Randall, S. Hardy, and L. Josefsson, Proc. Natl. Acad. Sci. USA, Vol. 75, pp. 1209-1212 (1978)).

S. Chan, et al., Proc. Natl. Acad. Sci. USA, Vol. 78, pp. 5401-5405 (1981) has exploited the ability of E. coli to remove the "pre" sequence. Chan, et al., modified cDNA for human preproinsulin to encode a hydrid "pre" sequence containing portions of E. coli and mammalian "pre" sequence. E. coli expressed the hydrid protein and correctly removed the "pre" sequence by intra-cellular processing. Thus, Chan, et al., was able to modify human preproinsulin cDNA in a way that would allow E. coli to produce proinsulin.

It is also known that yeast shares the ability to remove "pre" sequences from its own pre-proteins. Furthermore, when an E. coli preprotein was genetically engineered into yeast, pre-B-lactamase was processed to B-lactamase. (Roggenkamp, et al., Proc. Natl. Acad. Sci. USA, Vol. 78, No. 7, pp. 4466-4470 (1981)).

The above type of processing of preproteins, however, will not process to mature proteins many of the mammalian hormone precursors and many of the other interesting mammalian protein precursors in E. coli. These latter hormone and protein precursors contain a "pro" portion which is not processed by the enzymatic mechanism responsible for processing the "pre" portion of preproteins. As shown above, for example, the natural precursor for insulin, i.e. preproinsulin is processed in E. coli to form proinsulin.

Many investigators have been unable to express pre-proteins in yeast or E. coli, let

alone get processing. Expensive and time consuming, investigative efforts have focused almost exclusively on genetically eliminating the "pre" sequences and the "pro" sequences in attempting to express mature proteins without intermediates.

In several prior art approaches, the need for processing precursor proteins has been overcome. Insulin is the result of natural processing in human tissue involving cleaving two peptide chains, A and B, from the single large precursor preproinsulin and assembling the A and B chains to form the mature hormone insulin. The A and B chains are located within proinsulin and hence E. coli which processes preproinsulin to proinsulin does not produce the mature hormone insulin. An approach to obtaining mature insulin using E. coli employs chemically synthesized genes compatible with E. coli.

A double-stranded synthetic DNA-coding sequence for the insulin A chain was synthesized chemically from fundamental nucleotide units to yield the correct coding sequence. An extra amino acid (methionine) was added at one end. This end was fused to the bacterial gene for the enzyme B-galactosidase which results in accumulations of fused B-galactosidase-insulin-A-chain protein. This same procedure was repeated for the B-chain which resulted in the production of fused B-galactosidase-insulin-B-chain protein.

The fused proteins are insoluble in water and readily isolated from broken cells. The A and B chains of insulin are released from B-galactosidase at the extra methionine by cyanogen bromide cleavage and subsequently mixed together under conditions that allow formation of disulfide bonds between A

and B chains, yielding mature insulin. (W. Miller, J. of Pediatrics, Vol. 99, pp. 1-15 (1981); D. Goeddel, et al., Proc. Natl. Acad. Sci. USA, Vol 76, pp. 106-110 (1979)).

The above prior art approach overcomes the need for processing a precursor protein, but in turn requires processing of the fused B-galactosidase-insulin-A-chain and B-galactosidase-insulin-B-chain proteins to mature insulin. Moreover, chemical synthesis of the DNA coding sequences for A-chain and B-chain involves substantial costs, even when considering that the B-galactosidase-insulin-A-chain gene and B-galactosidase-insulin-B-chain gene after being synthesized are easily replicated for subsequent production of insulin. (D. Williams, et al., Science, Vol. 215, pp. 687-689 (Feb. 1982); W. Mahoney, Univ. of Wash. Medicine, supra).

The approach of chemically synthesizing DNA encoding for mature proteins has also been shown to be effective for bacterial production of human somatostation. (K. Itakura, et al., Science, Vol. 198, pp. 1056-1063 (1977)). However, insulin chains A and B and human somatostation are relatively small sequences and chemically synthesized DNA coded for them are relatively small. In the case of larger proteins, chemical synthesis of the DNA coding sequence coded for such proteins is prohibitively time consuming.

One prior art approach, now often followed, utilizes chemically synthesized DNA in conjunction with enzymatically prepared cDNA to produce a gene which instructs production of mature hormone in bacteria. Human growth hormone (HGH) is a protein of 191 amino acids, its precursor having an additional 26 amino acid "pre" portion. cDNA

encoding the precursor was enzymatically prepared from mRNA isolated from human pituitary tissue. The first useful cleavage site of the cDNA occurs at the site encoding amino acid residues 23-24 of HGH. Treatment of the cDNA with restriction endonuclease Hae III gives a DNA fragment of 551 base pairs which includes coding sequences for amino acids 24-191 of HGH. A gene fragment having coding sequences for residues 1-23 of HGH (and an initiation codon) was chemically synthesized. The two DNA fragments were combined to form a synthetic-natural hybrid gene which when inserted into a plasmid vector directed expression of mature HGH in E. coli. (D. Goeddel, et al., Nature, Vol. 281, pp. 544-548 (October 1979)).

Using a similar strategy of cleavage and reconstriction of DNA for the mature protein, R. Lawn et al., Nucleic Acids Research, Vol. 9, No. 22, pp. 6103-6114 (1981), expressed mature human albumin in E. coli.

This general approach, however, requires time consuming chemical synthesis of desired gene fragments, cleavage of cDNA assuming the availability of useful cleavage sites and difficult genetic construction of plasmids from DNA fragments. Furthermore, in both of the above examples, an initiator methionine was left at the $NH_2$ -terminal. The initiator methionines cannot practically be removed since HGH and albumin also have methionines located elsewhere in the sequence. Thus, removing the initiator methionine by cayanogen bromide cleavage, would result in cleavage at the other methionines. This would result in a protein split into cleaved fragments. Both the HGH and albumin produced by the above approach are "mature" proteins which start with methionines. Hence they

are not "real" mature proteins.

The prior art approaches set forth above illustrate that a major and nearly universal problem in producing mature proteins is the construction of the actual genetic material to be inserted into transfer vectors. Procedures exist for preparing cDNA from mRNA isolated from mammalian or other higher order animal tissue, but mammalian and higher order animal proteins are most often expressed as precursors and subsequently processed into the mature protein in cells of origin. The prior art has identified E. coli and yeast as microorganisms capable of processing precursors containing the "pre" portion, but this class of precursors excludes many of precursors of interest. The prior art thus has not identified a microorganism suitable for cloning mammalian and higher order animal genes which is capable of processing to mature proteins precursors of greatest interest. The prior art approaches attempt to solve the problem by constructing genes that code for mature protein. However, although procedures now exist for identifying nucleotide coding sequences for mature proteins, chemical synthesis of DNA sequences encoding mature proteins or fragments thereof for use in hydrid genes is costly and time consuming, often prohibitively so.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows inferred protein cleavage sites within the precursor of yeast α-factor, where "K" designates lysine and "R" designates arginine amino acid residues.

FIG. 2 shows the cDNA sequence encoding preproparathyroid hormone and the unique Pvu II and Hinf 1 cleavage sites.

FIG. 3 shows certain portions of the nucleotide sequence of the pYEM-1 plasmid.

## SUMMARY OF THE INVENTION

In the present invention, a method is disclosed for producing protein in yeast transformed to express a corresponding precursor containing a pair or triplet of basic amino acid residues located proximally and/or distally adjacent to the protein portion of the precursor sequence comprising proteolytic processing by the yeast of the precursor at the site of such pairs or triplets of basic amino acid residues. The method comprises proteolytic processing by transformed yeast which contains an endopeptidase, designated herein as a trypsin-like enzyme or enzymes. The trypsin-like enzyme or enzymes proteolytically process the precursor at the site of such pairs or triplets of basic amino acid residues by cleaving at the distal side of such pairs or triplets. The method further comprises proteolytic processing by transformed yeast that contains an exopeptidase, designated herein a carboxypeptidase-B-like enzyme or enzymes. The carboxypeptidase-B-like enzyme or enzymes proteolytically process the precursor at the site of such pairs or triplets of basic amino acid residues by degrading such pairs or triplets of basic amino acid residues remaining distally adjacent to the protein portion of the precursor sequence after the cleavage by the trypsin-like enzyme or enzymes.

In the present invention, the above method is further disclosed for proteolytic processing of proto-proteins to mature proteins. Proto-proteins, defined with greater specificity infra, consist generally of precursor proteins in which the

protein portion of the precursor sequence is identical in structure to the mature protein except for the abscence of the amino terminal and the carboxyl terminal in the precursor sequence. The above method is also disclosed for proteolytic processing of certain non-proto-proteins. For example, the above method is disclosed for proteolytic processing of preproinsulin or proinsulin to mature insulin. The above method is disclosed for producing mammalian insulin generally as well as human, bovine, and porcine insulin specifically. According to the method, preprocalcitonin and procalcitonin may be proteolytically processed by transformed yeast to form mature calcitonin or a calcitonin relative in the case of animal calcitonin generally and human, bovine, and porcine calcitonin specifically.

In the present invention, a recombinant DNA plasmid transfer vector useful for transforming yeast comprising a DNA sequence comprising the preproparathyroid gene cDNA sequence is disclosed as well as the plasmid pYEM-1 and yeast transformed by a plasmid comprising the above transfer vector and yeast transformed by the plasmid pYEM-1.

DESCRIPTION OF THE SPECIFIC EMBODIMENT

Proto-proteins may consist of precursors for which DNA and mRNA encoding the precursors naturally occur in animals. This type of proto-protein is designated source natural proto-proteins. Proto-proteins may also consist of precursors in which synthetic DNA encodes the precursor. This type of proto-protein is designated source synthetic proto-protein. For example, by chemical synthesis, or alternatively by enzymatic cleavage, rearrangement and subsequent fusion, DNA can be synthesized so that the precursor which

it encodes has the cleavage properties discussed below. Production of mature protein might be enhanced by transforming yeast with synthetic DNA encoded for a precursor having repetitive sequences of the mature protein, each sequence being flanked by appropriate cleavage sites.

Source natural proto-proteins are illustrated by, but not limited to, certain hormone precursors, including preproparathyroid (J. Habener & J. Potts, The New England Journal of Medicine (Second Part), Vol. 299, No. 12, pp. 635-643 (Sept. 1978)), preprosomatostatin (P. Hobart, et al., Nature, Vol. 288, pp. 137-139 (November 1980)), AVP-NpII precursor to arginine vasopressin and its corresponding neurophysin (H. Land, et al., Nature, Vol. 295, pp. 299-303 (January 1982)), cortitropin B-lipotropin precursor to corticotopin (ACTH) and B-lipotropin (B-LPH) (S. Nakanishi, et al., Nature, Vol. 278, pp. 423-427 (March 1979)), preproglucagon (P. Lund, et al., Pro. Natl. Acad. Sci. USA, Vol. 79, pp. 345-349 (January 1982)), and pro-opiomelanocortin (POMC) precursor to B-endorphin and Met- and Leu-enkephalin precursor (M. Comb, et al., Nature, Vol. 295, pp. 663-666, (February 1982)).

Source natural proto-proteins are also illustrated by melittin precursor (G. Suchanek, et al., Eur. J. Biochemistry, Vol. 60, pp. 309-315 (1975); G. Suchanek, et al., Proc. Natl. Acad. Sci. USA, Vol. 75, pp. 701-704 (1978)) and serum albumin precursors (R. Lawn, et al., Nucleic Acids Research, Vol 9, No.22, pp. 6103-6114 (1981)).

As reported in the above citations, these precursors contain within their sequence at least one mature protein sequence. Where there is a single mature protein sequence contained in the precursor it is flanked proximally by a pair or

triplet of basic amino acid residues consisting of lysine and/or arginine and is flanked distally by either the carboxyl-terminal of the precursor or a pair or triplet of basic amino acid residues lysine and/or arginine. If there are several mature protein sequences contained in the precursor, at least one of the mature protein sequences is flanked proximally by a pair or triplet of such basic amino acid residues and is flanked distally by either the carboxyl-terminal of the precursor or a pair or triplet of such basic amino acid residues. Any precursor protein falling within this description is defined herein as a proto-protein, whether it be source natural or source synthetic.

As reported in the above citations in connection with observing the production of mature proteins in mammals and other higher order animals, the cleavage site located on the distal side of a pair or triplet of such basic amino acid residues is readily attacked by endopeptidases with trypsin-like activity. After endopeptidase cleavage, any residual basic residues remaining adjacent to and on the distal side of the mature protein are susceptible to degrading, i.e. selective removal, by exopeptidases with activity resembling that of carboxypeptidase-B.

Thus, for example, in preproparathyroid hormone the mature protein is flanked proximally by the basic triplet lysine-lysine-arginine and is flanked distally by the carboxyl-terminal of the precursor. A single cleavage by a trypsin-like enzyme is sufficient to produce the mature hormone. In other proteins such as the glucagon precursor, two mature glucagon proteins are flanked both proximally and distally by a basic pair lysine-arginine. Combined cleavage by a trypsin-like

enzyme and degradation of the resulting carboxyl-terminal by a carboxypeptidase-B-like enzyme are required to produce the mature proteins.

The method of the present invention comprises preteolytic processing by yeast of proto-proteins to mature proteins. In the method, transformed yeast naturally containing a trypsin-like enzyme or enzymes and a carboxypeptidase-B-like enzyme or enzymes, proteolytically release mature proteins from larger precursors. These enzymes will effectively cleave and degrade proto-proteins to mature proteins. This is confirmed by a trypsin-like cleavage, discussed infra, of preproparathyroid hormone yielding mature parathyroid hormone. This is further confirmed by yeast processing its own mating factor, α-factor. (T. Tanaka, et al., J. Biochemistry, Vol. 82, pp. 1681-1687 (1977)). As shown in FIG. 1, the nucleotide sequence of α-factor shows that yeast naturally expresses a precursor containing four distinct codings for mature α-factor. Three of the four α-factors in the precursor are flanked distally by a pair of basic amino acids residues. A trypsin-like cleavage in combination with a carboxypeptidase-B-like degrading naturally yields correctly processed C-termini for these three α-factors. After a trypsin-like cleavage, N-termini of the four α-factors are flanked proximally by a series of several glutamic acid and alanine amino acid residues. These latter residues are in turn removed by an aminopeptidase. The foregoing natural endopeptidase and exopeptidase activity in yeast in combination with the virtual uniform presence of pairs and triplets of lysine and/or arginine flanking mature hormone sequences in proto-proteins underlies the present invention.

Although preproinsulin and proinsulin containing disulfide bonds are not proto-proteins as defined herein they will nevertheless undergo proteolytic processing in yeast transformed to express the preproinsulin or proinsulin. A pair or triplet of basic amino acid residues are located distally and/or proximally adjacent to the insulin-A-chain and the insulin-B-chain portions of the sequence which constitute the protein portion of the precursor preproinsulin and proinsulin sequence. The requisite disulfide bonds between the insulin-A-chain portion of the sequence and the insulin-B-chain portion of the sequence will be formed in yeast. (cf. the numerous examples of disulfide bond formation in yeast disclosed in M. Dayhoff, Atlas of Protein Sequence and Structure, Vol. 5 and Supplements 1, 2 & 3 (National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C. 20007 (1972, 1973, 1976, and 1981))). Proteolytic processing at the site of such pairs or triplets of basic amino acid residues will yield mature insulin from preproinsulin or proinsulin containing the disulfide bonds.

In the absence of disulfide bond formation between the insulin-A-chain portion of the sequence and the insulin-B-chain portion of the sequence, proteolytic processing will yield insulin-A-chain and insulin-B-chain, which may be caused in turn to attach to one another by disulfide bonds by conventional means to form mature insulin. In this case, the insulin-A-chain and insulin-B-chain may be considered mature proteins and preproinsulin and proinsulin without disulfide bonds may be considered a proto-protein according to the above discussion of proto-proteins.

Mature calcitonin contains disulfide bonds between the cysteines located at positions 1 and 7 of the sequence, contains a carbohydrate attached at the sequence at position 3, and the proline at position 32 has been amidated to pro-amide while the glycine at position 33 has been removed. Preprocalcitonin and procalcitonin will contain the requisite disulfide bonds. (cf. the numerous examples of disulfide bond formation in yeast as disclosed in Dayhoff, supra). A carbohydrate will be attached at position 3 in calcitonin. Preprocalcitonin and procalcitonin will undergo proteolytic processing in yeast transformed to express the preprocalcitonin or procalcitonin. A pair of basic amino residues are located proximally adjacent to the 33 amino acid sequence, while a triplet is located distally adjacent to the 33 amino acid sequence. It is expected that amidation of the proline located at 32 will occur in yeast after the cleavage distall to and degradation of the triplet. (cf. numerous examples of amidation in yeast as disclosed by Dayhoff, supra). In the event that a carbohydrate differing from the carbohydrate of mature calcitonin is formed by the yeast, the calcitonin relative containing the differing carbohydrate may be converted to mature calcitonin by conventional means. In the event that amidation following cleavage and degradation is suppressed, the calcitonin relative lacking the amidation may also be converted to mature calcitonin by conventional means.

By reverse transcription, cDNA can be prepared encoding any proto-protein of interest by isolating mRNA from tissues expressing the protein. Although many hormone and other protein genes have

already been cloned in E. coli, yeast has heretofore not been the host of choice. cDNA not previously cloned in yeast can be rendered compatible with a yeast host by proper codon selection (J. Bennetzen & B. Hall, J. Bio Chem., Vol. 257, pp. 3026 (1982)) and by site specific mutagenesis of the cDNA (G. Simmons, et al., Nucleic Acid Research, Vol. 10, pp. 821 (1982)).

Thus, one of the fundamental problems with producing useful mature proteins by recombinant DNA techniques has been simplified in the case of mature proteins derived from proto-proteins. cDNA, although readily available for most proteins by reverse transcription of mRNA isolated from animal tissue, will express the precursor of the mature protein. Yeast, but not E. coli, has the requisite enzymes to process expressed proto-proteins, preproinsulin, or proinsulin to mature protein or insulin.

## EXPERIMENTAL

In order to demonstrate the present invention, the following experiment was carried out.

The plasmid YEp-13 was obtained from Dr. Steven Henekoff, Fred Hutchinston, Dept. of Developmental Biology, Seattle, Washington, and can be constructed according to J. Broach, et al., Gene, Vol. 8, pp. 121-133, (1979). The gene which encodes yeast alcohol dehydrogenase 1 was modified according to Hitzelman, et al., Nature (London), Vol. 293, pp. 717-722 (1981), allowing the isolation of the transcription signals. These sequences, including the cloning site, were provided by Dr. G. Ammera. The plasmid YEp-13 was modified so that the $tet^R$ gene of YEp-13 was interrupted at the Bam Hl site with the yeast alcohol dehydrogenase 1 gene promotor and RNA polymerase

stop sequences. A Hind III site between the latter two elements provided the cloning site. These modifications of plasmid YEp-13 were accomplished by methods set forth generally in U.S. Patent 4,237,224, supra, and Methods of Enzymology, Vols. 65 and 68 wherein such methods are reviewed.

The cDNA sequence coding bovine preproparathyroid hormone, shown in FIG. 2 and further described in B. Kemper, et al., Hormonal Control of Calcium Metabolism (Ed. by D. Cohn, et al., published Excerpta Medica at Amsterdam, Oxford, and Princeton 1981) at pp. 19, was obtained from Dr. Byron Kemper, Department of Physiology and Biophysics and School of Basic Medical Sciences, University of Illinois-Urbana. This cDNA sequence was restricted with the enzymes PVU II and Hinf 1 at the sites shown in FIG.2. These enzymes were obtained from New England Biolaboratories, Beverly MA. The Hinf 1 site shown in FIG. 2 was filled with nucleotides using the enzyme DNA polymerase 1 (the large fragment) which was obtained from New England Nuclear, Boston, MA. This modified sequence was then blunt-end ligated to Hind III linkers and restricted with the enzyme Hind III. The Hind III linkers and Hind III enzyme were obtained from New England Biolaboratories, supra. The resulting DNA fragment was then ligated into the Hind III site of the modified plasmid YEp-13 forming a novel plasmid. This plasmid was designated pYEM-1. FIG. 3 shows certain portions of the nucleotide sequence of pYEM-1. The foregoing construction of pYEM-1 was accomplished by methods set forth generally in U.S. Patent No. 4,237,224, supra, the BLR M13 handbook, and Methods of Enzymology, Vols. 65 and 68 wherein such methods are

reviewed.

After constructing pYEM-1, yeast cells were transformed with the plasmid using the methods of Beggs, Nature (London), Vol. 275, pp. 104-109 (1978) and Hinnen, et al., Proc. Natl. Acad. of Sci. USA, Vol. 75, pp. 1929-1933 (1978). Because pYEM-1 has the yeast leu 2 gene, the use of a leu 2 negative strain of yeast was used in the transformation for the purposes of selecting successful transformants. Yeast strain, X1069-2D, a strain of Saccharomyces cerevisiae defective in leu-2 function, was obtained from the Yeast Genetic Stock Center, Univ. of Califorinia-Berekeley.

Of course any other defective yeast strain, including strains within Saccharomyces pombe and other species, could be used. All that is required is that a complementation system be established between the yeast strain and the cloning/expression vector and that the vector be stabily maintained in yeast. For example, a Trp 1 strain could be used if the Trp 1 gene was on the vector. To date, several stable transformation systems have been described. (A. Hinnen and B. Meyhack, Current Topics in Microbiology and Immunology, Vol. 96, pp. 101-117 (1981); C. Hollenberg, Current Topics in Microbiology and Immunology, Vol. 96, pp. 119-144 (1981)).

The transformed yeast cells containing plasmid pYEM-1 were grown in a leucine deficient media containing 5% glucose, yeast extract, yeast nitrogen base and other nutrients suitable for yeast strain X1069-2D. After 24 hours of growth at 30°C, the media was collected and the yeast cells lysed. Bioassay was performed according to conventional techniques and PTH

radioimmunoassay was performed using Immuno Nuclear Corporation (Stillwater, MN) assays specific to the N-terminal, mid-molecule, and C-terminal regions of parathyroid hormone. The following table shows that both immunologically cross-reactive parathyroid hormone and biologically active parathyroid hormone is being produced in yeast.

TABLE

| | PTH N-terminal RIA* | PTH Mid-molecule region RIA* | PTH C-terminal RIA* | Bioassay* |
|---|---|---|---|---|
| Cell lysate | | | | |
| pYEM-1 | 16 | 16 | 16 | 10 |
| control | 0 | 0 | 0 | 0 |
| Media | | | | |
| pYEM-1 | 2 | 2 | 2 | 0.015 |
| control | 0 | 0 | 0 | 0 |

*expressed in nanomoles/ml

To confirm that correct processing had occurred, 50 ml of culture was prepared in which the parathyroid hormone producing yeast were grown in media containing $^{35}S$ methionine (80 μ ci/ml). After an overnight growth the cells were removed by centrifugation. The media was then incubated with specific N-terminal parathyroid hormone antibody. After two hours the antibody-antigen complex was recovered by centrifugation and washed three times with new media followed by an ether wash. This complex contained about 7,000 cpm of $^{35}S$ methionine incorporated into protein after TCA precipitation. This mixture was applied to a Beckman 890D

sequencer according to the methods of Mahoney and Nute, Biochem. Vol. 19, pp. 4436 (1980) and subsequently degraded 40 cycles. Sequence analysis demonstrated that the $^{35}S$ methionine was all contained in cycles number 8 and 18. In mature PTH, methionine appears only at positions 8 and 18 in the sequence. If preproparathyroid hormone expressed by the yeast was left unprocessed, we would expect $^{35}S$ methionine in cycles 1, 2, 7, 11, 14, 49, and 59 reflecting the appearance of methionine at positions -31, -30, -25, -21, -18, +8, +18 in the preproparathyroid sequence.

The novel microorganism yeast strain X1069-2D transformed by novel plasmid pYEM-1, designated X1069-2D-pYEM-1, was placed on permanent deposit in the Northern Regional Research Center, U.S. Dept. of Agriculture, Peoria, Illinois 61604 on September 8, 1982. The NRRL number for X1069-2D-pYEM-1 is Y-15153. The plasmid pYEM-1 and the transfer vector contained therein may be removed from this novel yeast strain by known means.

While the invention has been described in connection with a specific embodiment thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations uses, or adaptations of the invention within the scope of the appended claims.

CLAIMS

1. A method for producing protein in yeast transformed to express a corresponding precursor containing a pair or triplet of basic amino acid residues located proximally and/or distally adjacent to the protein portion of the precursor sequence, comprising proteolytic processing by the yeast of the precursor at the site of such pairs or triplets of basic amino acid residues.

2. The method of claim 1 wherein the proteolytic processing by yeast of the precursor at the site of such pairs or triplets of basic amino acid residues comprises cleaving, by a trypsin-like enzyme or enzymes present in the transformed yeast, at the distal side of such pairs or triplets of basic amino acid residues.

3. The method of claim 2 wherein the proteolytic processing by yeast of the precursor at the site of such pairs or triplets of basic amino acid residues further comprises degrading, by a carboxy-peptidase-B-like enzyme or enzymes present in the transformed yeast, of any such pairs or triplets of basic amino assay residues remaining distally adjacent to the protein portion of the precursor sequence after the cleavage by the trypsin-like enzyme or enzymes.

4. The method of claim 1 wherein the corresponding precursor is a proto-protein.

5. The method of claim 4 wherein the proto-protein is source synthetic proto-protein.

6. The method of claim 4 wherein the proto-protein is source natural proto-protein.

7. The method of claim 6 wherein the source natural proto-protein is bovine preproparathyroid hormone.

8. The method of claim 1 wherein the protein is mammalian insulin and the corresponding precursor is mammalian preproinsulin or proinsulin.

9. The method of claim 8 wherein the mammalian insulin and mammalian preproinsulin or mammalian proinsulin are members respectively of the group consisting of human insulin and human preproinsulin or human proinsulin, bovine insulin and bovine preproinsulin or bovine proinsulin, and porcine insulin and porcine preproinsulin or porcine proinsulin.

10. The method of claim 1 wherein the protein is animal calcitonin or an animal calcitonin relative and the precursor is animal preprocalcitonin or animal procalcitonin.

11. The method of claim 10 wherein the animal calcitonin or animal calcitonin relative and the animal preprocalcitonin or animal procalcitonin are members respectively of the group consisting of human calcitonin or human calcitonin relative and human preprocalcitonin or human procalcitonin, bovine calcitonin or bovine calcitonin relative and bovine preprocalcitonin or bovine procalcitonin, and porcine calcitonin or porcine calcitonin relative and porcine preprocalcitonin or pro-calcitonin.

12. The method of claim 1 wherein the yeast is Saccharomyces cerevisiae or Saccharomyces pombe.

13. The method of claim 12 wherein the yeast is Saccharomyces cerevisiae.

14. A recombinant DNA plasmid transfer vector useful for transforming yeast comprising a DNA sequence comprising the preproparathyroid gene cDNA sequence.

15. The plasmid pYEM-1

16. Yeast transformed by a plasmid comprising the transfer vector of claim 14.

17. Yeast transformed by the plasmid of claim 15.

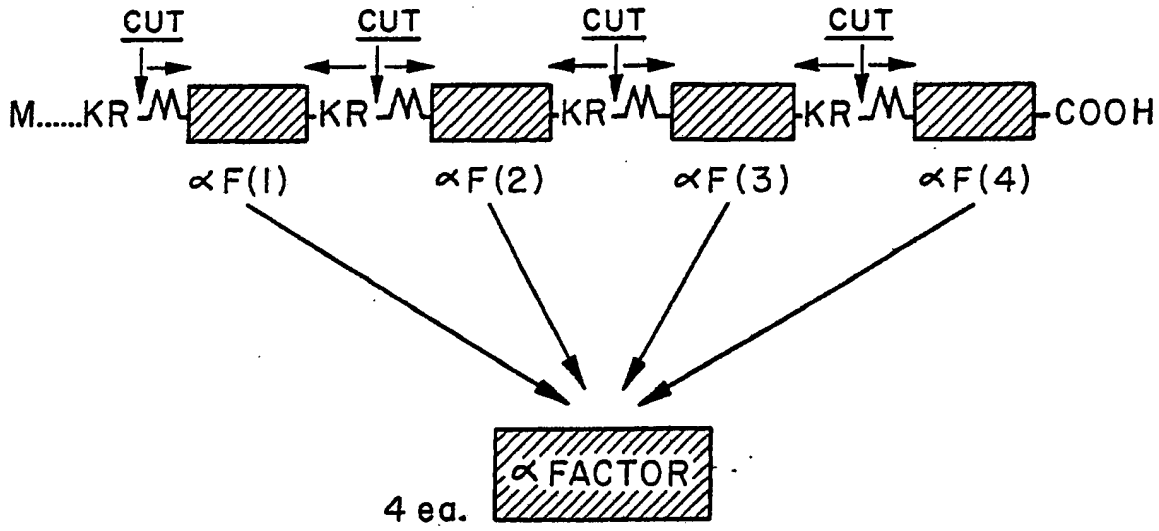


*Fig 1*

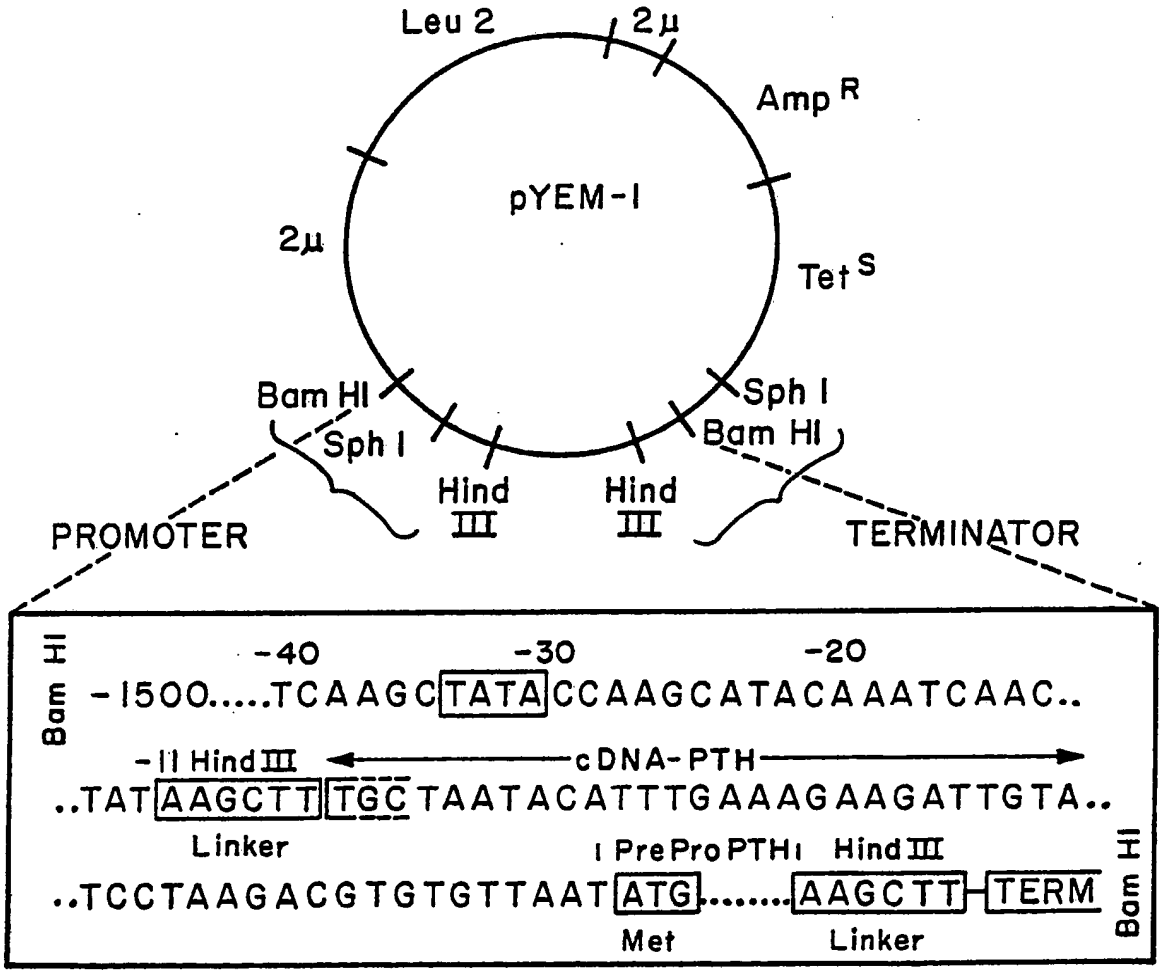


*Fig 3*

*PvuII Cleavage Site C/T*

```
5' G GGG GGG GGG GGG GGG GGG GTT TTA TCA GCC TTC TCA GGT TTA CTC AAC TTT GAG AAA GCA TCA GCT GCT AAT ACA TTT
                10          20          30          40          50          60          70

                                                  met met ser ala lys asp met val lys val met ile val met leu
GAA AGA AGA TTG TAT CCT AAG ACG TGT GTT AAT ATG ATG TCT GCA AAA GAC ATG GTT AAG GTA ATG ATT GTC ATG CTT
80          90          100         110         120         130         140         150

ala ile cys phe leu ala arg ser asp gly lys ser val lys lys arg ala val ser glu ile gln phe met his asn leu
GCC ATC TGT TTT CTT GCA AGA TCA GAT GGG AAG TCT GTT AAG AAG AGA GCT GTG AGT GAA ATA CAG TTT ATG CAT AAC CTG
  160         170         180         190         200         210         220         230

gly lys his leu ser ser met gly arg val glu trp leu arg lys lys leu gln asp val his asn phe val ala leu gly
GGC AAA CAT CTG AGC TCC ATG GAA AGA GTG GAA TGG CTG CGG AAA AAG CTA CAG GAT GTG CAC AAC TTT GTT GCC CTT GGA
 240         250         260         270         280         290         300         310

ala ser ile ala tyr arg asp gly ser ser gln arg pro arg lys lys glu asp asn val leu val glu ser his gln
GCT TCT ATA GCT TAC AGA GAT GGT AGT TCC CAG AGA CCT CGA AAA AAG GAA GAC AAT GTC CTG GTT GAG AGC CAT CAG
320         330         340         350         360         370         380         390

lys ser leu gly glu ala asp lys ala asp val asp val leu ile lys ala lys pro gln stop
AAA AGT CTT GGA GAA GCA GAC AAA GCT GAT GTG GAT GTA TTA ATT AAA GCT AAA CCC CAG TGA AAA CAG ATA TGA TCA GAT
  400         410         420         430         440         450         460         470

CAC TGT TCT AGA CAG CAT AGG GCA ACA ATA TTA CAT GCT GCT AAT GTG TTC ACC TTC TAT TAA GTG CCA GTA GTT CTA TGA
 480         490         500         510         520         530         540         550
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*Hinf Cleavage Site G/A*

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CCA ACC TTT ATT GCT AGC TGT GAT ACC TAC AAT TTT AAT TGA GTA TTT TGA TTC TAC TTT ATT CAT CTA AGA GCT CTT
560         570         580         590         600         610         620         630

TTA ATA ATT CTA TTT CTA TTG ATT CCA AAT AAA TGA AGT TAA GTA TTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
  640         650         660         670         680         690         700         710

AAA AAA AAA AAA AAA AAA CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC CCC 3'
 720         730         740         750         760
```

**Fig 2**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US83/01361

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL[3] C12P21/00,C12N15/00,1/18,1/00

U.S. CL. 435/68,172,256,317

## II. FIELDS SEARCHED

Minimum Documentation Searched [4]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/68,172,256,317 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

CHEMICAL ABSTRACTS FILES 308,309,310,320 and 311
BIOSIS FILES 5,55 and 255

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

| Category * | Citation of Document, [16] with indication, where appropriate, of the relevant passages [17] | Relevant to Claim No. [18] |
|---|---|---|
| Y | N, WILLIAMSON, GENETIC ENGINEERING 4 ACADEMIC PRESS PP108-125,1983. | 1-17 |
| A | N, WALTON, RECOMBINANT DNA ELSEVIER SCIENTIFIC PUBLISHING CO., PP.185-197 and 213-227. | 1-17 |
| A | GB, A 2068969 A, PUBLISHED 19 AUGUST 1981. | 1-17 |

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search [2] | Date of Mailing of this International Search Report [2] |
|---|---|
| 25 NOVEMBER 1983 | 06 DEC. 1983 |
| **International Searching Authority [1]** | **Signature of Authorized Officer [20]** |
| ISA/US | ALVIN E. TANENHOLTZ |

Form PCT/ISA/210 (second sheet) (October 1981)